MEDICAL LIBRARY

VOLUME 37 NUMBER 4

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

APRIL, 1939

EDITOR-IN-CHIEF
C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR

Published Monthly by THE WILLIAMS & WILKINS COMPANY
MT. ROYAL AND GUILFORD AVES., BALTIMORE, U. S. A.

Copyright, 1939, The Williams & Wilkins Company

Made in United States of America

Sustaining Members

Society of American Bacteriologists

Abbott Laboratories	North Chicago, Ill.
A. S. Aloe Company	St. Louis, Mo., and Los Angeles, Cal.
American Instrument Company	Silver Spring, Md.
American Sterilizer Company	Erie, Pa.
Anheuser-Busch, Inc.	St. Louis, Mo.
Bausch & Lomb Optical Company	Rochester, N. Y.
Braun-Knecht-Heimann Company	San Francisco, Cal.
Central Scientific Company	Chicago, Ill.
Clay-Adams Company, Inc.	New York, N. Y.
Coca-Cola Company	Atlanta, Ga.
Cutter Laboratories	Berkeley, Cal.
Difco Laboratories, Inc.	Detroit, Mich.
Eimer and Amend	New York, N. Y.
J. B. Ford Company	Wyandotte, Mich.
Gilliland Laboratories	Marietta, Pa.
Hynson, Westcott & Dunning	Baltimore, Md.
Kimble Glass Company	Vineland, N. J.
Lederle Laboratories	New York, N. Y.
E. Leitz, Inc.	New York, N. Y.
Eli Lilly & Company	Indianapolis, Ind.
Mead Johnson and Company	Evansville, Indiana
National Aniline & Chemical Company, Inc.	New York, N. Y.
Parke, Davis & Company	Detroit, Mich.
Pfaltz & Bauer, Inc.	New York, N. Y.
Redman Scientific Company	San Francisco, Cal.
Scanlan-Morris Company	Madison, Wisc.
Sheffield Farms Company, Inc.	New York, N. Y.
Spencer Lens Company	Buffalo, N. Y.
Arthur H. Thomas Company	Philadelphia, Pa.
Will Corporation	Rochester, N. Y.
Wilmot Castle Company	Rochester, N. Y.
Carl Zeiss Inc.	New York, N. Y.

Professional Directory Society of American Bacteriologists

THE Council of the Society has authorized the establishment of a directory where *those who desire to fill vacancies* may secure a selected list of applicants. This list is composed of teachers of bacteriology, technicians in laboratories, applicants for fellowships and scholarships in bacteriology serology, immunology, and research workers in medical or agricultural bacteriology. Employers may receive information regarding applicants without charge. Information regarding vacancies should be filed upon a blank which has been prepared to secure uniform information regarding a particular vacancy.

Apply for application blanks a. the following address: F. M. Meader, M.D., *Detroit Department of Health, Detroit, Mich.*

Employment Agency Society of American Bacteriologists

THE Council of the Society has authorized the establishment of an employment agency. Bacteriologists and technicians may learn of positions by making application to the address below. Applicants for positions if not members of the Society will agree to send to the agency 5% of their salary for the first three months. If the applicant is a member of the Society he will agree to send only 3% of his salary for the first three months.

All funds accruing to the Society in excess of necessary and actual expenses, after being audited by the Society will be transferred to the treasurer of the Society for the improvement and enlargement of its Journal, support of its research fellowship, collection of cultures of bacteria, etc.

Send for application blanks to F. M. Meader, M.D., *Detroit Department of Health, Detroit, Mich.*

Entered as second-class matter April 17, 1916 at the postoffice at Baltimore, Maryland, under the Act of March 3, 1879.
Subscription price $5.00 per volume. Single numbers, $1.00.

THE LACTASE ACTIVITY OF ESCHERICHIA COLI-MUTABILE[1]

CHARLES J. DEERE, ANNA DEAN DULANEY AND I. D. MICHELSON

Department of Chemistry and Department of Bacteriology, University of Tennessee School of Biological Sciences, Memphis

Received for publication August 24, 1938

In a previous publication (Deere, Dulaney and Michelson, 1936) we reported that the non-lactose-fermenting (white) form of *Escherichia coli-mutabile* when grown in lactose broth utilizes very small quantities of lactose, if any, before the appearance of lactose-fermenting variants (reds). Appreciable lactose utilization and production of acid and gas were so closely correlated with the appearance of lactose-fermenting variants as to warrant the conclusion that the fermentative changes were due to these variants.

This earlier study has been extended to include determinations of the lactase content of cell preparations of both the white and red forms of *Escherichia coli-mutabile*. Since both forms are able to ferment monoses the difference in their cultural behavior in lactose broth must be due to a difference either in lactase content or activity.

The behavior of *Escherichia coli-mutabile* in lactose broth suggests that its lactase is an adaptive enzyme (Virtanen, 1934), viz., that lactase is produced only in the presence of lactose. Hershey and Bronfenbrenner (1936) found the non-lactose-fermenting form of a lactose-variable coli-aerogenes intermediate to be *deficient* in lactase, while its lactose-fermenting variant contained lactase. We were surprised, therefore, to find that

[1] Part of the data in this paper are taken from a dissertation presented by Charles J. Deere to the Committee on Graduate Study in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Tennessee, September, 1937.

lactase in *Escherichia coli-mutabile* is not an adaptive enzyme, in terms of Virtanen's definition, since its presence is not dependent on growth of the organism in the presence of lactose (tables 2 and 3). Furthermore, the lactase activity of this organism in cultures is not determined directly by its lactase content.

The isolation and cultural reactions of the organism used in this study have been described (Dulaney and Michelson, 1935 and Michelson and Dulaney, 1936). The Garrett strain used in former work was employed in these studies. The white is the non-lactose-fermenting *Escherichia coli-mutabile* and the red is its lactose-fermenting variant.

METHODS

Analytical

The lactase content of cell preparations was determined by their ability to hydrolyze lactose, the change in reducing power of the substrate being measured by the Shaffer-Hartmann method as modified by Shaffer and Somogyi (1933). With Reagent No. 50 and a 15 minute period of heating, the per cent increase in reducing power of a solution of lactose follows closely the per cent of hydrolysis of lactose (fig. 1).

Cell preparation

Determination of lactase content by the above method requires cell preparations whose ability to act on monoses has been destroyed. Karstrom (1931) and Hershey and Bronfenbrenner (1936) have used toluene for inhibiting enzyme action on monoses and preventing the interference of contaminants. We have found that thymol will sterilize heavy suspensions of colon organisms within one hour, while toluene requires 2 days (Hershey and Bronfenbrenner). In other respects thymol is also more satisfactory than toluene.

In order to measure accurately the test quantities of cell preparations we have weighed samples dried by vacuum distillation at 35–38°C., having demonstrated that this procedure is innocuous to lactase.

Substrate

The substrate used was 50 cc. of 0.5 per cent lactose in 1 per cent acacia and 0.1 M phosphate buffer at pH 7.0–7.2. The presence of acacia prevents lactase inactivation by unknown factors which otherwise prevent even roughly agreeing duplicates, particularly when the quantities of cell preparations are varied.

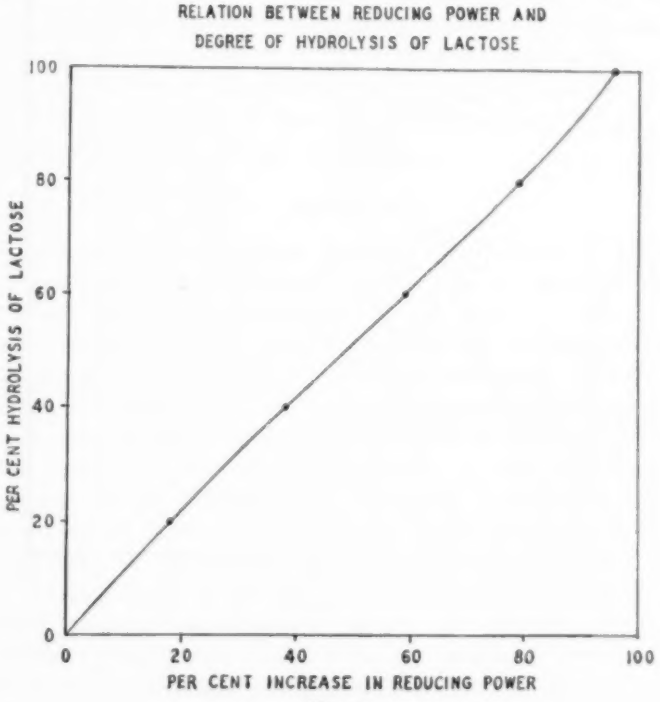


FIG. 1

The pH of the substrate is optimum for the lactase of this organism (fig. 2). For sugar determination no preliminary treatment of the system is required; the digests are simply diluted to bring the sugar content within the range determinable by the reagent.

pH determination

We have used a quinhydrone electrode for pH determinations.

Time of appearance of variants

The time of appearance of variants was determined by streaking the cultures on Endo's agar.

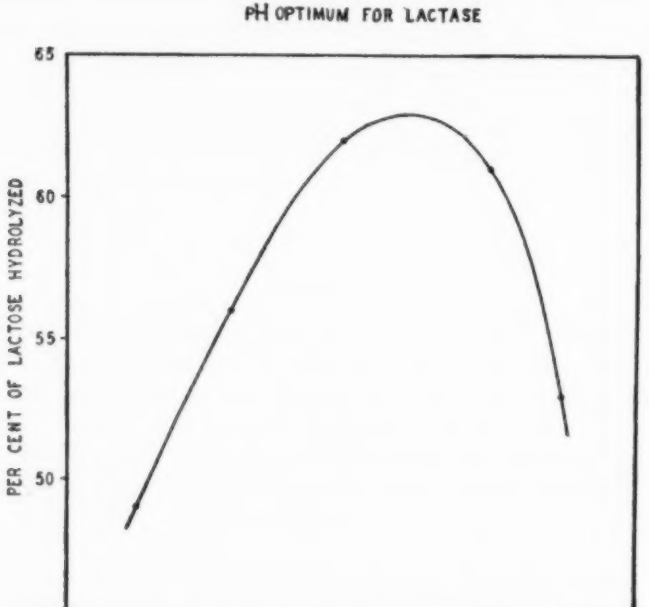


FIG. 2

EXPERIMENTAL PROCEDURE

The organisms were grown on plain or sugar agar contained in flatsided pint flasks. Ten-per-cent sugar solutions, separately sterilized, were used in the sugar agar. The growth was harvested in 8–12 cc. of saline, the cells packed by centrifugation, and the supernatant fluid used for pH determination. The cells were washed twice in distilled water and samples estimated to contain 20–30 mgm. of cells placed in weighed 100 cc. Erlenmeyer

flasks. The samples were dried rapidly by vacuum distillation with the apparatus described by Morrison and Hisey (1937) and the weight of dried material determined. The dried cells were suspended in 25 cc. of 2 per cent acacia in 0.2 M phosphate buffer, 10–20 mgm. of thymol added, and the samples placed in an incubator at 37–38°C. After periods ranging from one to one and one-half hours, 25 cc. of 1 per cent lactose were added and samples immediately withdrawn for analysis. These samples and those subsequently taken were diluted with 0.01 per cent copper sulfate in order to stop enzyme action. The reducing power of these diluted samples was determined and thereby the lactase content of the preparation estimated.

RESULTS

In our earlier work (Deere, Dulaney and Michelson, 1936) on lactose utilization of *Escherichia coli-mutabile* we employed broth cultures. Since it was desirable to use agar cultures for the present enzyme studies, a comparison of the lactase content of cells grown on the different media was indicated. The results (table 1) disclose a relatively delayed increase of lactase in cells grown on agar, and a more rapid decrease in lactase of cells in broth cultures following development of lactose-fermenting variants. These differences in behavior, however, are slight enough to justify use of agar cultures for our present purposes.

Our observations were extended to include a determination of the lactase content of cell preparations of both the white and red strains when grown on plain, glucose and lactose agar. The results are presented in tables 2 and 3. It will be observed that growth on lactose medium is not essential for the presence of lactase in either of the strains. Incidentally, the white strain used in this experiment had not been in contact with lactose for 55 days. When grown on lactose-free media the two strains have similar small amounts of lactase. Growth of either strain on lactose medium yields preparations which are many times as active as preparations obtained from lactose-free media. The white strain grown on lactose agar has about 5 times the lactase content of the red strain which has been grown on lactose-free

agar. The lactase content of the red strain when grown on lactose rises to approximately 3 times that of the white under the same conditions. The fact that a growing culture of the white strain will not ferment lactose cannot be attributed to absence of lactase.

In view of these results it seemed unlikely that increase in lactase could be the entire explanation for the development of red variants. Probably a combination of factors, rather than a single one, is responsible. The quantitative increase in lactase which occurs cannot be ignored. It may be that there is a

TABLE 1

Comparison of lactase activity of cells grown in broth and on agar

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1. Period of incubation in determination of lactase activity, 12 hours

	MEDIUM																
	Lactose agar						Plain agar				Lactose broth					Plain broth	
Period of growth, hours.	12	24	48	72	120	240	12	24	48	72	12	24	48	72	120	24	48
pH of medium	7.2	7.3	6.5*	6.3	7.4	7.9	7.5	7.6	7.9	7.9	7.4	7.6	5.1*	4.9		7.6	7.9
Weight of sample, mgm.	24.8	22.2	18.8	19.2	17.0	23.5	27.0	25.3	25.5	25.1	25.4	18.0	20.6	20.0	18.3	18.5	22.4
Per cent of lactose hydrolyzed.....	18	15	48	87	63	47	3	3	1	6	23	14	91	72	1	2	0
Lactase activity†..	0.7	0.7	2.5	4.5	3.7	2.0	0.1	0.1	0	0.2	0.9	0.8	4.4	3.6	0	0.1	0

* Lactose-fermenting variants appeared at this interval.

† Per cent of lactose hydrolyzed per milligram of dry preparation.

critical lactase content which must be achieved before sugar utilization and acid formation sufficient to change the dye-indicator system on Endo's agar results. This critical value is acquired by the red strain during the very early hours of the growth phase. On the other hand, only certain cells of the white strain are able to ferment lactose, even after prolonged (48 hour) contact with the sugar.

Very small amounts of lactose are sufficient to cause the development of a few reds. Since the lactase content of these organisms is so greatly influenced by the presence of lactose in the medium it seemed likely that small concentrations of lactose

could cause the production of reds without increasing the lactase activity of the cells. This possibility was examined in the

TABLE 2

Effect of medium on lactase activity of Escherichia coli-mutabile (Garrett white)

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1. Period of incubation in determination of lactase activity, 12 hours

	MEDIUM											
	Glucose agar				Lactose agar				Plain agar			
Period of growth, hours	12		24		12		24		12		24	
pH of medium	4.7		4.6		7.2		7.2		7.6		7.7	
Treatment of cells	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried
Weight of dry sample, mgm.	30.6		19.8		34.7		28.7		29.9		23.0	
Per cent of lactose hydrolyzed	4	6	2	4	36	37	30	30	7	4	3	4
Lactase activity*	0.1	0.2	0.1	0.2	1.0	1.1	1.0	1.0	0.2	0.1	0.1	0.2

* Per cent of lactose hydrolyzed per milligram of preparation.

TABLE 3

Effect of medium on lactase activity of Escherichia coli-mutabile (Garrett red)

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1. Period of incubation in determination of lactase activity, 12 hours

	MEDIUM											
	Glucose agar				Lactose agar				Plain agar			
Period of growth, hours	12		24		12		24		12		24	
pH of medium	4.7		4.7		5.1		5.2		7.5		7.7	
Treatment of cells	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried
Weight of dry sample, mgm.	32.4		23.5		32.1		24.7		38.0		23.6	
Per cent of lactose hydrolyzed	0	4	5	3	90	86	70	68	4	6	7	4
Lactase activity*	0	0.1	0.2	0.1	2.8	2.7	2.8	2.8	0.1	0.2	0.3	0.2

* Per cent of lactose hydrolyzed per milligram of preparation.

following experiments (table 4). Garrett white was grown on agar containing 0.001, 0.01, 0.1 and 1 per cent lactose and the lactase content of the cells followed. Lactose in concentration of

0.01 per cent was sufficient to cause the production of red variants but this concentration gave a negligible increase in lactase content over that observed in organisms grown on 0.001 per cent of lactose-free agar, on which reds failed to appear. These results may indicate that increase in lactase content of the white strain is not as closely correlated with variation from white to red as we first believed (Dulaney and Deere, 1937). On the other hand, it may be argued that the particular cells of the white strain which gave rise to the reds did so by increasing their lactase content. It is well known that a great variation in rate of dissociation may be demonstrated among colonies which represent

TABLE 4

Effect of lactose concentration on lactase activity of Escherichia coli-mutabile

Substrate—0.5 per cent lactose, 1 per cent acacia, 0.1 M phosphate, pH 7.1. Period of incubation in determination of lactase activity, 12 hours

	LACTOSE CONCENTRATION IN MEDIUM											
	0.001 per cent			0.01 per cent			0.1 per cent			1.0 per cent		
Period of growth, hours	24	48	72	24	48	72*	24	48*	72	24	48*	72
pH of medium	7.6		8.1	7.6	7.7	8.0	7.6	7.6	7.4	7.4	6.9	6.4
Weight of dry sample, mgm.	26.1		26.3	25.4	27.5	26.8	28.8	28.9	23.2	28.5	27.4	28.9
Per cent of lactose hydrolyzed	6		5	6	8	7	9	21	37	28	35	51
Lactase activity†	0.2		0.2	0.2	0.3	0.3	0.3	0.7	1.6	1.0	1.3	1.8

* Lactose-fermenting variants appeared at this interval.

† Per cent of lactose hydrolyzed per milligram of dry preparation.

the descendants of one white colony. Furthermore, a few reds may be demonstrated in broth containing 0.0025 per cent lactose, though this concentration of lactose does not yield sufficient acid to produce a color change with brom-thymol-blue indicator.

The white strain may be grown on lactose agar for an indefinite period of time without giving rise to reds, provided frequent (daily) transfers are made. Under these conditions the lactase content of the cells approaches but never equals that of reds grown under the same conditions.

CONCLUSIONS

1. Lactase is present in both the white and red forms of *Escherichia coli-mutabile* when these organisms have been grown

in the absence of lactose. Lactase must therefore be classed as a constitutive enzyme, though the cultural behavior of the organisms would lead one to class its lactase as an adaptive enzyme.

2. Growth of both the white and red organisms on lactose media results in preparations several times as active in lactase as similar preparations obtained from lactose-free media. Under such conditions the lactase content of the white approaches, but never equals, that of reds grown under similar conditions.

3. The white form can be kept in contact with lactose indefinitely without the production of red forms, in spite of marked increase in lactase.

4. Dissociation to reds may occur in small degree without a measurable increase in lactase content of the entire culture.

5. Preparations of white organisms grown on lactose contain more lactase than preparations of red organisms grown in the absence of lactose.

6. Quantitative increase in lactase does not altogether explain dissociation which is probably due to a combination of factors.

REFERENCES

DEERE, CHARLES J., DULANEY, ANNA DEAN, AND MICHELSON, I. D. 1936 The utilization of lactose by *Escherichia coli-mutabile*. Jour. Bact., **31**, 625–633.

DULANEY, ANNA DEAN, AND DEERE, CHARLES J. 1937 Studies on *Escherichia coli-mutabile* in relation to lactose utilization. Jour. Bact., **33**, 19.

DULANEY, ANNA DEAN, AND MICHELSON, I. D. 1935 A study of *B. coli-mutabile* from an outbreak of diarrhea in the new-born. Amer. Jour. Pub. Health, **25**, 1241–1251.

HERSHEY, A. D., AND BRONFENBRENNER, J. 1936 Dissociation and lactase activity in slow lactose-fermenting bacteria of intestinal origin. Jour. Bact., **31**, 453–464.

KARSTRÖM, H. 1931 Zur Spezifität der a-Glucosidasen. Biochem. Z., **231**, 399–403.

MICHELSON, I. D., AND DULANEY, ANNA DEAN 1936 Comparative study of *B. coli-mutabile* from an outbreak of diarrhea in the new-born. South. Med. Jour., **29**, 611–617.

MORRISON, DEMPSIE B., AND HISEY, ALAN 1937 The preparation of hemoglobin in a dry and active state. Jour. Biol. Chem., **117**, 693–706.

SHAFFER, PHILIP A., AND SOMOGYI, MICHAEL 1933 Copper iodometric reagents for sugar determination. Jour. Biol. Chem., **100**, 695–713.

VIRTANEN, ARTURRI I. 1934 On the enzymes of bacteria and bacterial metabolism. Jour. Bact., **28**, 447–460.

A NOTE ON THE MONOPHASIC NON-SPECIFIC SALMONELLA TYPES[1]

D. W. BRUNER AND P. R. EDWARDS.

Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Ky.

Received for publication September 2, 1938

The discovery of phase variation by Andrewes (1922) has had a profound effect on the differentiation of *Salmonella* types. This phenomenon and the demonstration of heat-stable and heat-labile receptors by Weil and Felix (1920) form the basis for the modern classification of the paratyphoid group. Through the work of a number of investigators it has gradually been accepted that the motile *Salmonella* strains are divisible into four groups. These are as follows:

A. Diphasic strains possessing the specific and non-specific phases of Andrewes (1922).

B. Diphasic cultures which display a variation confined to two specific antigens. This is the alpha-beta phase variation of Kauffmann and Mitsui (1930).

C. Monophasic cultures which exist only in the specific phase.

D. Monophasic cultures which exist only in the non-specific phase. These are usually considered as the non-specific counterparts of diphasic cultures.

It is with the fourth group, the forms generally regarded as being totally and permanently non-specific, that the present paper deals. This group is composed of five varieties: Binns, Kunzendorf, Voldagsen, Berlin and Puerto Rico. It is proposed to demonstrate that these organisms are not totally non-specific but are actually diphasic and possess specific components.

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

METHODS

The simplest method of isolating a specific phase from a *Salmonella* strain is to plate the culture and examine the resulting colonies by slide agglutination with specific and non-specific serums. Such a procedure is quite satisfactory in strains which produce a relatively large percentage of specific colonies, but is most laborious and quite likely to result in failure when the culture under examination produces very few specific colonies. Scott (1926) and Bridges and Scott (1931) isolated specific phases from stubbornly non-specific cultures by cultivating them for several generations in broth containing antiserum derived from the non-specific phase. After this treatment, specific colonies appeared on plate cultures.

Wassén (1935) described a method for the separation of specific and non-specific phases which was based on the immobilizing effect of homologous immune serum. Filter paper impregnated with the appropriate serum was placed in semi-solid agar and the medium inoculated at a point somewhat removed from the paper.

The migration of the homologous phase was checked at a distance from the paper, while the heterologous phase approached more closely. This method was improved and simplified by Gard (1937), who added the immune serum directly to semi-solid agar and inoculated the medium by stabbing. The growth of the homologous phase was confined to the line of stab while the heterologous phase diffused through the medium. This modification was used by the writers. The details of the method are as follows: Approximately 5 ml. of the semi-solid agar of Jordan, Caldwell and Reiter (1934) was placed in tubes having a diameter of 11 mm. After sterilization, the medium was cooled to 45°C. and sufficient sterile agglutinating serum derived from the Kunzendorf type added to give a final concentration of 1 to 1200. The serum had a titre of 1 to 5000 for the homologous strain. The concentration of serum must be gauged rather carefully since too little fails to confine the growth of the homologous phase to the line of stab, while too large an amount of serum immobilizes both phases through the action of somatic

agglutinins. The agar containing the non-specific serum was inoculated by a single stab at one side of the tube. A heavy line of growth appeared along the line of stab and if organisms existing in the specific phase were present they grew out through the medium, forming protuberances from the line of inoculation. By fishing from the protruding growth and reinoculating into the same medium the specific phase was obtained. At times it was necessary to transfer the culture in this manner several times before the non-specific phase was eliminated. It is helpful to transfer the culture to an agar slant also, each time it is transferred in semi-solid agar. Growth from the agar slant can be tested by slide agglutination with specific and non-specific serums, thus enabling the worker to judge the status of the culture at each transfer.

RESULTS

The Binns type of Schütze (1920) was described by White (1926) as a totally non-specific variant of *Salmonella typhimurium*. It was found by Edwards (1936) to be diphasic. The specific phase of the orignal Binns strain was demonstrable only with the greatest difficulty by ordinary plating methods and by growth in non-specific antiserum broth. Therefore, it offered an opportunity to compare the efficiency of those methods with that of the Wassén technique. When the Binns strain was inoculated into the semi-solid agar containing non-specific serum, the whole tube of agar was turbid after over-night incubation. It was first thought that the concentration of serum was insufficient to confine the non-specific phase to the site of inoculation. However, when organisms were fished from the side of the tube opposite the line of stab, they were found to represent the specific phase and failed to agglutinate with non-specific antiserum. This was repeated several times and each time the result was the same. The results with the Binns strain demonstrate how much more effective is the Wassén technique than are other methods of isolating specific components.

The Kunzendorf variety, or western European type of *Salmonella cholerae-suis*, was found by White (1926) to be totally

non-specific. Gard (1937) mentions the fact that he obtained specific phases from Kunzendorf cultures by the Wassén technique. The two cultures to be reported upon here are type cultures (numbers 309 and 1713) of the National Collection of Type Cultures of Great Britain. These two strains were among those studied by White and found monophasic. When they were inoculated into the test medium, both displayed a small spherical projection from the line of stab after 48 hours incubation. On the second transfers the migrations from the site of inoculation were much more pronounced and on the third transfers the specific phase was isolated from both strains. These specific phases were agglutinated to the titre of specific *S. cholerae-suis* antiserum, were not flocculated by non-specific serums and were able to absorb completely the floccular agglutinins from specific *S. cholerae-suis* antiserum. In addition to the two cultures described, specific phases were also isolated from 6 other Kunzendorf strains.

The Voldagsen type of Dammann and Stedefeder (1910) was demonstrated by White (1926) to be the monophasic non-specific variant of *Bacillus typhi-suis* of Glässer (1909). Serologically, it is identical with the Kunzendorf type but differs in its very delicate growth on artificial mediums and by its rapid fermentation of arabinose and trehalose. The only strain of this type available was the Dammann strain of the National Collection of Type Cultures of Great Britain. Four tubes of semi-solid agar were inoculated with the culture. After 48 hours incubation one of them exhibited evidence of phase variation. After four transfers, subcultures from this tube yielded the specific phase. It was agglutinated to the titre of specific *S. cholerae-suis* antiserum and was not flocculated by non-specific serums.

The Berlin type was described by Kauffmann (1929) as a totally non-specific variant of the Thompson type of Scott (1926). Gard (1937) recorded the isolation of specific phases from Berlin cultures. Four cultures were available for study, 3078 of the National Collection of Type Cultures of Great Britain and three cultures received from Dr. Kauffmann. The specific phase of culture 3078 was isolated after three transfers in semi-solid agar. The remaining three cultures behaved as

did the Binns strain, the specific phase clouding the whole tube on the first transfer in the semi-solid medium. The specific phases were flocculated to the titre of specific Thompson antiserum but were not agglutinated by non-specific serums. The specific phase of the Berlin type removed all agglutinins from specific Thompson antiserum.

The Puerto Rico type was described by Kauffmann (1934) as a totally non-specific variant of the Newport type. Only one culture, the strain originally studied by Kauffmann, was available. When it was inoculated into semi-solid agar the non-specific phase spread through the medium and no specific components could be isolated. The amount of serum in the medium was then doubled and the specific phase was isolated without difficulty. The specific phase was flocculated to the titre of specific Newport antiserum, did not agglutinate with non-specific serums and removed all agglutinins from specific Newport antiserum when used in absorption tests.

DISCUSSION

The demonstration of specific components from cultures of all the so-called monophasic non-specific varieties of *Salmonella* makes extremely doubtful the existence of a true permanently and totally non-specific strain. The presence of specific phases in the organisms under discussion renders it no longer necessary to include the Binns, Voldagsen, Puerto Rico and Berlin varieties in the classification of the genus *Salmonella*. They are identical with *Salmonella typhi-murium*, *Salmonella typhi-suis*, the Newport type and the Thompson type respectively. The Kunzendorf type is serologically identical with *Salmonella cholerae-suis* but differs in its biochemical activity.

The technique of Wassén is far superior to other methods generally used for the isolation of specific phases from stubbornly non-specific races. Kauffmann (1936) discovered that the typhoid bacillus, which was long regarded as a monophasic organism, actually displays alpha-beta phase variation. It seems quite possible that by the use of this method other supposedly monophasic specific species may be found to exhibit phase variation.

CONCLUSIONS

The Wassén technique is an excellent means of isolating phases of *Salmonella* which are suppressed under ordinary conditions of culture. By its use specific phases have been isolated from all of the so-called monophasic non-specific *Salmonella* types.

REFERENCES

ANDREWES, F. W. 1922 Studies in group agglutination. I. The Salmonella group and its antigenic structure. Jour. Path. Bact., **25**, 505–524.

BRIDGES, R. F., AND SCOTT, W. M. 1931 A new organism causing paratyphoid fever in India, Salmonella, type Bareilly. Jour. Roy. Army Med. Corps, **56**, 241–250.

DAMMANN AND STEDEFEDER. 1910 Untersuchungen über Schweinepest. Archiv. f. wiss. u. prakt. Tierheilk., **36**, 432. Abstr. in Zbt. Bakt., Abt. I., Ref., 1911, **49**, 139–141.

EDWARDS, P. R. 1936 Serological variants of Salmonella *typhimurium* with special reference to *S. typhimurium* var. *binns*. Jour. Hyg., **36**, 348–354.

GARD, S. 1937 Ein Colistamm mit Salmonella-H-Antigen, zugleich ein Beitrag zur Frage der Definition der Salmonellagruppe. Zeitschr. f. Hyg., **120**, 59–65.

GLÄSSER, K. 1909 Zum heutigen Stande der Schweinepestfrage und zu den weiteren Untersuchungen von Uhlenhuth, Hübener, Xylander und Bohtz über das Wesen und die Bekämpfung der Schweinepest und über die Bakteriologie der Hogcholera (Paratyphus B)—Gruppe, D. tierartzl. Wschr. **17**, 513–516 and 529–534.

JORDAN, E. O., CALDWELL M. E., AND REITER, D. 1934 Bacterial motility. Jour. Bact., **27**, 165–174.

KAUFFMANN, F. 1929 Zur Paratyphusfrage. Zbt. Bakt. Abt. I, Ref., **94**, 282–288.

KAUFFMANN, F. 1934 Zur Serologie des Newport Bazillus. Zbt. f. Bakt., Abt. I., Orig., **132**, 161–163.

KAUFFMANN, F. 1936. Über die diphasische Natur der Typhusbacillen. Zeitschr. f. Hyg., **119**, 104–118.

KAUFFMANN, F., AND MITSUI, C. 1930 Zwei neue Paratyphustypen mit bischer unbekannten Phasenwechsel. Zeitschr. f. Hyg., **111**, 740–752.

SCHÜTZE, H. 1920 The paratyphoid B group. Lancet, **1**, 93–97.

SCOTT, W. M. 1926 The "Thompson" type of Salmonella. Jour. Hyg., **25**, 398–405.

WASSÉN, A. 1935 Essais d'application au vibrion cholérique de la méthode fondée sur la faculté de déplacement des bactéries. Bull. mensuel de l'Office Internatl. d'Hyg. publ., **25**, 1–14.

WEIL, E., AND FELIX, A. 1920 Über den Doppeltypus der Rezeptoren in der Typhus-Paratyphus-Gruppe. Zeitschr. f. Immunitätsf., **29**, 24–91.

WHITE, P. B. 1926 Further studies of the Salmonella group. Med. Res. Counc. Gt. Brit., Sp. Rpt. Ser. No. 103.

PRODUCTIVITY OF MEDIA CONTAINING MILK FOR RECENTLY ISOLATED STRAINS OF THE COLIFORM GROUP[1]

M. T. BARTRAM[2] AND L. A. BLACK

Department of Bacteriology, University of Maryland, College Park

Received for publication September 3, 1938

The productivity of various media used in water analysis for the isolation of the coliform group has been frequently reported in recent literature. Since such studies may not be applicable where milk constitutes a portion of the inoculum, the Most Probable Number was determined in these experiments in a series of liquid and solid media containing milk.

The method described by Butterfield (1933) and Hoskins (1933), for determining the comparative productivity of media for coli-aerogenes organisms, was modified, in that 10 instead of 15 tubes of each medium were used and two dilutions were planted instead of three. Sterile whole milk in 1 cc. amounts was added to each tube of liquid media, except standard lactose broth, before the organisms were added. With solid media the number of organisms in the buffer infusion was determined by plating in standard agar. From the results obtained after 24 hours' incubation, two dilutions of the original suspension were plated into each of the trial media in triplicate, such dilutions being used that the plates would show between 30 and 300 colonies per plate. Sterile whole milk was added to each plate in 1 cc. quantities before introducing the suspension.

The media employed were selected from those used by us in a

[1] Based on a portion of a thesis submitted by the senior author to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address, Bacteriological Section, Food Division, Food and Drug Administration, United States Department of Agriculture, Washington, D. C.

previous study (Bartram and Black, 1936) and were prepared from dehydrated products or according to the methods described by their proponents. Both the liquid and solid media were made up in such strengths that the addition of the 1 cc. of milk would serve to reduce the concentration of the medium to the usual

TABLE 1

Productivity of media containing milk for coliform organisms; results reported as percentage of most probable numbers compared to standard lactose as 100 per cent

(Average of two recently isolated strains)

MEDIA	ESCHERICHIA	AEROBACTER	INTERMEDIATE	AVERAGE
Methylene-blue brom-cresol-purple	99	107	34	80
Fuchsin lactose	71	50	79	66
Brilliant-green bile	75	81	36	64
Formate ricinoleate	15	36	27	26
Eijkman	0	0	0	0

TABLE 2

Productivity of media containing milk for coliform organisms; results reported as percentage of colonies obtained compared to standard agar plus milk as 100 per cent

(Average of two recently isolated strains)

AGAR	ESCHERICHIA	AEROBACTER	INTERMEDIATE	AVERAGE
Neutral-red bile	97	121	146	121
Violet-red bile	120	92	128	113
Endo	86	116	103	102
Brilliant-green lactose bile	75	91	120	95
Taurocholate	93	123	30	82
Desoxycholate	92	70	20	61
Trypaflavin	4	45	5	18

value. All tubes and plates were examined at the end of 24 hours and 48 hours. Two strains each of *Escherichia, Aerobacter* and intermediates recently isolated from milk were used. The dilutions were so prepared that 1 cc. quantities could be used in making each inoculation and in all instances the media were inoculated alternately.

RESULTS

The results obtained are shown in tables 1 and 2, in which the percentage productivity of each liquid medium is based on standard lactose broth as 100 per cent and each solid medium on standard agar plus milk as 100 per cent. On the basis of the results obtained, the liquid media would be placed in the following order, the most productive first: (1) methylene-blue brom-cresol-purple; (2) fuchsin lactose; (3) brilliant-green bile, and (4) formate-ricinoleate. The Eikjman medium was unsatisfactory, giving negative results in all tests with the dilutions used. The solid media would rank as follows: (1) neutral-red bile; (2) violet-red bile; (3) Endo; (4) brilliant-green lactose bile; (5) lactose taurocholate; (6) desoxycholate, and (7) trypaflavine agar.

DISCUSSION

These experiments may not be sufficiently extensive to evaluate completely the various media and for such a purpose a more detailed examination may be necessary, as pointed out by Noble (1935). However, the results may be used within recognized limits, especially since no "border-line" medium was indicated. The results are somewhat in disagreement with those obtained in water analysis by other workers, none of whom employed milk with the inoculum. Ruchhoft (1935) in summarizing data received from coöperating laboratories, using the method of Butterfield-Hoskins, obtained the following productivity from highest to lowest: buffered lactose, fuchsin lactose, methylene-blue brom-cresol-purple, brilliant-green bile, crystal violet and formate-ricinoleate. Ruchhoft and Norton (1935) in a preliminary report obtained essentially the same results except that the positions of buffered lactose and fuchsin lactose were reversed. Farrell (1935) rated buffered lactose and brilliant-green bile, in the order named, ahead of fuchsin lactose and placed trypaflavin broth below formate-ricinoleate. In this laboratory, Black and Klinger (1936) observed buffered lactose, fuchsin lactose, methylene-blue brom-cresol-purple, brilliant-green bile, crystal-violet and formate-ricinoleate to rank in the order named. These results agree with

those found by Ruchhoft. It would be interesting to determine if the addition of 1 cc. of milk caused the change in relative productivity of fuchsin lactose and methylene-blue brom-cresol-purple.

As far as is known, the productivity of the various solid media has not been reported so that no comparison is possible. However, the results in general confirm those obtained in previous work by us, (Bartram and Black, 1936) although desoxycholate agar fell below the results that might have been expected. This was accounted for by its low productivity with the strains of the intermediate group and may be characteristic of the medium, although this has not been determined with additional intermediate strains.

SUMMARY

Two strains each of *Escherichia*, *Aerobacter*, and intermediates recently isolated from raw milk were inoculated into five liquid and seven solid media in addition to standard lactose broth and standard methods agar, employed as controls.

A modification of the method of Butterfield was followed with two dilutions inoculated into 10 tubes of each medium. Two dilutions were plated in triplicate in the solid media and 1 cc. of sterile whole milk was added to each plate or tube before adding the organisms.

The productivity of the liquid media tested, based on Hoskins' Most Probable Numbers was, in order of descending sensitivity: (1) methylene-blue brom-cresol-purple; (2) fuchsin lactose; (3) brilliant-green bile; (4) formate-ricinoleate; (5) Eijkman medium. No one of the media was as productive as standard lactose.

The solid media ranked as follows: (1) neutral-red bile; (2) violet-red bile; (3) Endo; (4) brilliant-green lactose bile; (5) lactose taurocholate; (6) desoxycholate, and (7) trypaflavine agar. The unsatisfactory results obtained with desoxycholate agar may be accounted for by its low productivity with the cultures of the intermediate group used. Neutral-red bile and violet-red bile gave counts considerably higher than those obtained on standard agar plus milk.

REFERENCES

BARTRAM, M. T., AND BLACK, L. A. 1936 Detection and significance of the coliform group in milk. I. A comparison of media for use in isolation. Food Research, **1**, 551–563.

BLACK, L. A., AND KLINGER, MARY E. 1936 A comparison of media for the detection of *Escherichia-aerobacter*. Jour. Bact., **31**, 171–179.

BUTTERFIELD, C. T. 1933 Comparison of the enumeration of bacteria by means of solid and liquid media. Pub. Health Repts., **48**, 1292–1297.

FARRELL, M. A. 1935 Comparative productivity tests of presumptive test media with pure cultures of the *Coli-aerogenes* group. Jour. Bact., **30**, 445.

HOSKINS, J. K. 1933 The most probable number of *B. coli* in water analysis. Jour. Am. W. W. Assoc., **25**, 867–877.

NOBLE, R. E. 1935 The relative productivity of certain culture media. Jour. Am. W. W. Assoc., **27**, 1143–1160.

RUCHHOFT, C. C. 1935 Comparative studies of media for the determination of the *Coli-aerogenes* group in water analysis. Jour. Am. W. W. Assoc., **27**, 1732–1745.

RUCHHOFT, C. C., AND NORTON, J. F. 1935 Study of selective media for *Coli-aerogenes* isolation. Jour. Am. W. W. Assoc., **27**, 1134–1142.

CHEMICAL FACTORS INFLUENCING THE GROWTH OF TUBERCLE BACILLI[1]

I. Metal Catalysts

BEN C. SHER AND HENRY C. SWEANY

Research Laboratory of the City of Chicago Municipal Tuberculosis Sanitarium

Received for publication September 17, 1938

INTRODUCTION

This investigation deals with the effects of cupric, manganous, ferric, and antimonate ions; extracts of calves' lungs, calves' hearts, and chicken livers; and of diphenylamine and other organic compounds; on the growth of H37, a human strain of tubercle bacilli. It was carried out at the suggestion of Dr. Kharasch, who, with his co-workers (Kharasch, Conway and Bloom, 1936), had studied the effects of the metal ions of copper, iron, and manganese as well as other compounds and had observed the response of the organisms in question toward these reagents.

To permit the greatest possible control of the factors involved, the bacilli were grown on Long's synthetic medium, (Long and Seibert, 1926) from which the ferric ammonium citrate was omitted. Long's medium is prepared by autoclaving a solution of the following in one liter of distilled water:

	gm.
Asparagin	5
Ammonium citrate	5
KH_2PO_4	3
Na_2CO_3	3
$MgSO_4 7H_2O$	1
NaCl	2
Glycerol	50
Ferric ammonium citrate	0.05

[1] Presented before the Division of Biological Chemistry of the American Chemical Society at the Pittsburgh meeting, September, 1936.

The solution is then adjusted to a pH of 7.2–7.4 with hydrochloric acid.

The ferric ammonium citrate, or other iron salts, when used in this investigation are considered as additional substances, in the same sense as are cupric or manganous salts.

It is important to note the composition of this medium in order to evaluate the effects of the constituents on metal catalysis. Kharasch, *et al.*, (Kharasch, Legault, Wilder and Gerard, 1936) have shown, in the case of the oxidation of a simple thiol system to dithiol, that the rate of oxidation is accelerated by the addition of manganese, copper, or iron salts and that their effectiveness is in the order listed. The acceleration due to the manganese is suppressed, and that due to copper catalysis is enhanced, by phosphate ions, to a degree depending upon the concentration and upon whether the phosphate ion is present as ortho-, meta-, or pyrophosphate. Glycerol inhibits the catalytic effect of iron, manganese, and copper ions. The carbonate ion probable does not affect these systems. If there is any effect, it is unchanged with different concentrations. It should also be noted that beef extract was found to inhibit the effect of iron and that liver extract inhibited both iron and copper catalysis, while the catalytic effect of manganese was not inhibited by either of these factors. The presence of dithiol in the system at the start hastened the oxidation of the thiol systems.

Similar effects are undoubtedly important in controlling the growth of bacteria on a solution such as Long's medium. However, the complexity of such a solution, particularly after it is autoclaved, or after bacterial growth has started, obviates any *a priori* statement as to the influence of additional substances on the growth of the bacilli. It is not known in what forms the phosphate ion is present, nor whether glycerophosphates are formed from the glycerol in the presence of these phosphates, nor what is the nature of the complexes which are formed from ammonium citrate, asparagin and glycerol. It has been determined that with traces of additional substances, there is a profound quantitative change and also a marked effect on the character of the growth of certain organisms. For example, the growth of

Chlorella vulgaris, on a synthetic medium (Meier, 1932) of inorganic salts, arginine and agar, which ordinarily does not take place in the presence of minute traces of copper, was unimpaired when liver extract was added to such a medium which contained copper (Kharasch, Conway and Bloom, 1936). The resultant abundant growth was dark orange instead of green, and, microscopically, the relatively large green alga had changed into a small spherical body. These striking changes caused Dr. Kharasch to suggest the study of bacteria under similarly modified conditions of growth.

PURIFICATION OF REAGENTS

For this investigation, the media are divided into two classes. In one, the chemicals used are "C.P." or "Reagent" grade (hereafter to be called "C.P." media) and in the other, the chemicals are purified to the extent that the traces of impurities are reduced to a practical minimum (hereafter called "Purified" media).

The water used for recrystallizing the reagents, for cleansing the glassware, and for the media was double distilled. The second distillation was carried out in an all "pyrex" glass still.

Asparagin was recrystallized four times from boiling water. The combined filtrates yielded upon evaporation a green-colored crop of asparagin which indicates the probable presence of chlorophyl-like impurities.

Potassium dihydrogen phosphate was recrystallized three times from boiling water.

Sodium carbonate was prepared by fusing purified sodium bicarbonate. The sodium bicarbonate was recrystallized three times from water between 50° and 60°C.

Ammonium citrate was purified in earlier experiments by recrystallizing the ammonium citrate twice from water. In subsequent experiments, citric acid was recrystallized and gaseous ammonia, washed through a train of three wash bottles, was passed through the solution of redissolved crystals.

Magnesium sulfate was recrystallized once from water.

Sodium chloride was prepared by passing purified hydrogen

chloride into a solution of sodium chloride. The precipitated salt was washed with water and dried.

Glycerol was distilled under reduced pressure from an all "pyrex" still.

Agar agar was used wherever solid media were required. For purified reagents, the agar was washed several times with water, and most of the water removed each time. The washed agar was then dialyzed in a cellophane bag for two days against double distilled water.

All the glassware used in preparing the media, the media flasks, and culture tubes were of "pyrex" glass. All glassware, in the earlier experiments, was cleaned with chromic-sulfuric acid cleaning solution. In later experiments, trisodium phosphate was used. In either event, after soaking 24 hours in the cleaning solution, the apparatus was washed thoroughly with hot tap water, and with distilled water and then allowed to stand in distilled water twice for 24-hour periods. The containers were plugged with cotton, then sterilized in an autoclave at 15 pounds steam pressure.

EXPERIMENTAL RESULTS

In several series of cultures, the growths were more abundant and appeared earlier on "C.P." media than on each similar modification of medium prepared with purified reagents. The "Purified" media required the addition of other substances to yield such abundant and accelerated growths. This demonstrates that the catalytic effect of the impurities present in "C.P." and reagent grade or similar quality chemicals is sufficiently great to influence and mask the effects of the traces of substances of interest in this investigation.

Table 1 was compiled from three series of cultures (H37) in each of which there were several flasks of the same modification of media. A number of interesting facts are brought out by this study. The effects of the non-ferrous catalysts on purified media, singly or in combination with each other, were not decisive. The addition of ferric ions alone did not promote growth, but the addition of ferric ions to any of the media containing the

non-ferrous catalysts singly, or in combinations, resulted in a remarkably enhanced growth. Therefore, the non-ferrous catalysts appear to have stimulated the action of the iron. It is

TABLE 1

The influence of metal catalysts on growth of tubercle bacilli (H37)

"PURIFIED"* MEDIA NUMBER	Cu	Mn	$K_4Sb_2O_7$, (H_2O)	Fe	GROWTH‡
	mgm. per 1000 cc.	*mgm. per 1000 cc.*	*mgm. per 1000 cc.*	*mgm. per 1000 cc.*	
1					1
2	0.033				1
3		0.10			1
4			30.0		2
5	0.033	0.10			1
6	0.033		30.0		2
7		0.10	30.0		2
8	0.033	0.10	30.0		2
9				7.5	1
10	0.033			7.5	3
11		0.10		7.5	3
12			30.0	7.5	5
13	0.033	0.10		7.5	4
14	0.033		30.0	7.5	5
15		0.10	30.0	7.5	5
"C.P."† MEDIA NUMBER					
16					3
17	0.033				4
18		0.10			4
19			30.0		4
20	0.033	0.10			4
21	0.033		30.0		5
22		0.10	30.0		5
23	0.033	0.10	30.0		4

* "Purified" media were prepared from reagents which were carefully purified.

† "C.P." media were prepared from "C.P." or reagent grade chemicals.

‡ The growths were evaluated by inspection, and classified into five groups. Five (5) represents the most abundant growth observed, and one (1) the sparsest growth.

consistent, then, that "C.P." or unpurified medium required no additional substance to yield enhanced growths, since minute traces of iron and other substances are already present in this

TABLE 2

The influence of metal catalysts on growth of tubercle bacilli (H37)

"PURIFIED" MEDIA NUMBER	Cu	Mn	$K_2Sb_2O_7$, (H_2O)	Fe	NUMBER OF FLASKS	AVERAGE WEIGHT OF GROWTH*	RANGE OF pH*
	mgm. per 1000 cc.	*mgm. per 1000 cc.*	*mgm. per 1000 cc.*	*mgm. per 1000 cc.*			
1					8	0.18	5.4 6.2
2	0.03				4	0.18	5.0 6.2
3		0.10			4	0.19	5.2 5.8
4			20.0		4	0.14	5.0 5.4
5	0.03	0.10			4	0.19	5.0 5.6
6	0.03		20.0		4	0.18	5.0 5.4
7		0.10	20.0		4	0.15	5.2 5.6
8	0.03	0.10	20.0		4	0.23	5.4 6.0
9				7.5	4	0.46	7.2 7.4
10				15.0	5	0.41	7.2 7.6
11	0.03			7.5	5	0.41	7.0 7.2
12	0.03			15.0	7	0.39	7.0 7.6
13		0.10		7.5	3	0.48	7.4
14		0.10		15.0	5	0.45	7.2
15			20.0	7.5	5	0.44	7.2 7.4
16			20.0	15.0	5	0.44	7.2 7.4
17	0.03		20.0	7.5	4	0.44	7.2 7.4
18	0.03		20.0	15.0	10	0.40	7.0 7.4
"C.P." MEDIA							
19					9	0.47	5.4 6.0

* The determinations of weights and pH were made five months after inoculation of the media. The "mother" culture was grown on Long's synthetic media for 17 days.

medium as impurities. It is noteworthy, although not indicated in the tabulations, that, where potassium pyroantimonate is present, growths appeared earlier and were more abundant than on similar media containing no pyroantimonate. This is also true of manganese salts although the effect is less marked.

It will be seen in table 2 that the weights of the growths obtained on "C.P." media and on "Purified" media containing iron, were more than twice the weight obtained from the analogous iron-free "Purified" media. It is to be recalled from table 1 that "Purified" medium containing iron and no other additional substance did not consistently yield enhanced growths. These differences may be attributed to some variations, either in technique or the introduction of minute traces of other metals.

No attempt was made in this study to investigate the pH-time relationship of the cultures, but some pH determinations which are of interest were made at the end of certain periods of growth. Five months after inoculation, "Purified" media which contained iron showed a pH which ranged from 7.0–7.6, while the pH range of the iron-free media was from 5.4–6.2. That of "C.P." media was from 5.4–6.0 (table 2).

In another series, the results were different, in that the pH of the substrates from a 10½ weeks' growth on "Purified" medium was 7.2 regardless of whether iron was added or not. In this latter series, the substrates from the "C.P." media varied from 5.4–6.0.

It is generally accepted that when a different medium is to be used in culturing bacteria, in order to obtain good growths, it is frequently necessary to allow the bacteria to adapt itself to the new environment. It is conceivable then, that the history of growth and the subsequent nature of the cultures used in transplanting may in some way be responsible for the differences observed. Thus, the cultures indicated in table 2 were transplanted from a 17-day old growth on Long's synthetic medium, while those indicated in table 3 were made from a 15-week old culture grown on Long's synthetic medium.

In order to substantiate whether differently grown cultures of the same strain yielded different results, the following experi-

ments were carried out. A culture of H37, which was grown on "Purified" media for 18 weeks, was used to prepare two subcultures. For one of these, "Purified" media was used (flask *a*); for the other, "C.P." media, to which iron solution was added, (flask *b*). These subcultures were allowed to grow for four weeks and were then used for transplanting on the following media: "Purified" alone, "Purified" containing iron, "C.P." alone, and "C.P." medium to which iron had been added. In each instance, the cultures from flask *b* yielded earlier growths, and, interestingly, the pH values of the solutions in these flasks were lower than those from flask *a* (table 4).

TABLE 3

The pH of cultures when inoculated from a "mother" culture 15 weeks old

"PURIFIED" MEDIA NUMBER*	Fe ADDED	NUMBER OF FLASKS	RANGE OF pH†
	mgm. per 1000 cc.		
1	0	5	7.2
9	7.5	7	7.2
"C.P." MEDIA NUMBER			
19	0	3	5.8-6.0
19 Fe	7.5	4	5.4-6.0

* The media used were reserved from the experiments described in table 2.

† The determination of pH were made 2½ months after inoculation of the media. The "mother" culture was grown on Long's synthetic media for 15 weeks.

The following experiments also show that there is a difference due to the type of inoculum used. Two emulsions of B599 (a nonpathogenic, rapidly growing bovine strain of tubercle bacilli) were prepared with a mechanical emulsifier devised in our laboratory and described by Corper and Cohen, (1936). One emulsion, (Emulsion A) was prepared from a 2-week old culture grown on "C.P." agar medium, and the other, (Emulsion B) was prepared from a 3-day old culture which had been grown on "Purified" agar medium through two generations. These were used to seed variations of "Solid Purified" medium which were prepared by adding 2 per cent of purified agar to "Purified" medium in which different concentrations and combinations of the following sub-

stances were incorporated: ferric ammonium citrate, manganous sulfate, vitamin C, calves' lung extract, calves' heart extract, chicken liver extract, ethylenediamine, and copper sulfate. These variations were prepared in quadruplicate and were then divided into two sets of duplicates. The first set was inoculated with Emulsion A, the second set with Emulsion B. *In each instance, growths appeared earlier in the first set.*

The addition of ferric ammonium citrate or other substances, did not promote growth on this agar medium, but when copper sulfate was used as the additional substance, it was found that

TABLE 4

Influence of growth history of culture on growth and pH of subcultures (H37)

"PURIFIED" MEDIA NUMBER	Fe	NUMBER OF FLASKS SEEDED FROM FLASK *a*	NUMBER OF FLASKS SEEDED FROM FLASK *b*	GROWTH AT 28TH DAY	pH
	mgms. per 1000 cc.				
1a		4		2	7.1
1b			3	5	6.9
2a	7.5	4		3	7.3
2b	7.5		3	4	6.0
"C.P." MEDIA					
3a		3		4	7.2
3b			4	6	6.8
4a	7.5	3		5	6.9
4b	7.5		4	6	6.0

The culture of H37 in flask *a* was grown on "Purified" media, that in flask *b* was grown on "C.P." media, to which iron was added. Each was inoculated from the same culture, which was grown on "Purified" media for 18 weeks.

in the first set, the initial growth was retarded and ultimate growth impaired at concentrations of 1:24,000 to 1:48,000, while there was no growth at all with a concentration of 1:6,000. In the second set, copper sulfate inhibited only the initial growth, while ultimate abundant growths were obtained with a concentration of 1:3,000, the highest concentration used.

These data prove that the history of the growth of the "mother" cultures of tubercle bacilli is most important.

In these experiments, we also used diphenylamine as an additional substance, and have observed that a concentration of

1:20,000 prevented growth of tubercle bacilli. A concentration of 1:135,000 retarded initial growth and yielded ultimate sparse growths. This is of particular interest because similar effects were obtained by Kharasch, *et al.* (Kharasch, Conway and Bloom, 1936) in the case of *Staphylococcus aureus*, *Pseudomonas pyocyanea*, *Serratia marcescens*, *Torula rosea*, *Sarcina lutea*, *Spirillum rubrum*, and *Aspergillus niger*, on the different media they used.

No attempt was made to study the morphology of the bacilli, their pathogenicity, or immunological effect in the present work, and no study was made of the products elaborated. The primary object was to make a preliminary survey to determine where consistently decisive and controllable trends could be obtained. It was our experience that many factors, only some of them controllable, had an effect on the growth of tubercle bacilli. The manner in which diphenylamine exerted its effect, however, is positive irrespective of the extraneous and inherent factors involved. Its inhibitory action was decisive (although quantitatively different) with all of the media used in this investigation. This is of interest; and further results obtained with diphenylamine and similar compounds will appear in a subsequent paper.

SUMMARY

1. Long's synthetic medium which was prepared from "C.P." or "Reagent" grade chemicals, and to which no iron was added, yielded more than twice as much growth of H37 as "Purified" Long's medium, which was prepared from purified reagents.

2. The addition of iron salts to "C.P." medium, i.e., Long's synthetic medium from which iron was omitted, promoted growth only slightly.

3. Non-ferrous catalysts added to "Purified" medium did not promote growth. The addition of iron salts to "Purified" medium, particularly in conjunction with copper, manganese, or pyroantimonate ions, either singly or in combinations, yielded more than a twofold enhancement of growth of H37, as compared with "Purified" media alone.

4. Copper sulfate in minute traces retarded initial growth, and in concentrations greater than 1:3,000, prevented growth altogether.

5. Potassium pyroantimonate yielded earlier and more abundant growths.

6. Manganous sulfate yielded similar results.

7. The pH of the substrates of the cultures and the early appearance of growth appear to be controlled by the growth history of the inoculating culture as well as the nature of the media used.

8. Diphenylamine retarded initial growth in high concentrations around 1:150,000, and prevented ultimate growth in concentrations greater than 1:20,000 in all media used.

REFERENCES

Corper, H. J., and Cohen, M. L. 1936 A mechanical device for preparing fine suspensions of tubercle bacilli and other micro-organisms. The Journal of Laboratory and Clinical Medicine, **21**, 4, 428–432.

Kharasch, M. S., Conway, E. A., and Bloom, W. 1936 Some chemical factors influencing growth and pigmentation of certain micro-organisms. Journal of Bacteriology, **32**, 5, 533–540.

Kharasch, M. S., Legault, Wilder, and Gerard 1936 Metal catalysts in biologic oxidation. I. The simple system: thioglycolic acid, buffer, metal, dithiol. Journal of Biological Chemistry, **113**, 2, 537–555; II. Tissue inhibition. Journal of Biological Chemistry, **113**, 2, 556–569.

Long, E. R., and Seibert, F. B. 1926 The chemical composition of the active principle of tuberculin. I. A non-protein medium suitable for the production of tuberculin in large quantity. American Review of Tuberculosis, **13**, 393–397.

Meier, F. E. 1932 Lethal action of ultraviolet light on a unicellular green alga. Smithsonian Miscellaneous Collections, **87**, 1.

IMMUNOLOGICAL RELATIONSHIPS OF POLYSACCHARIDES OF MUCOID ORGANISMS OF THE TYPHOID-SALMONELLA GROUP

H. R. MORGAN AND T. D. BECKWITH

Department of Bacteriology, University of California at Los Angeles

Received for publication September 27, 1938

The work of Furth and Landsteiner (1928), (1929); White (1929), and Boivin, Mesrobeanu and Mesrobeanu (1933a), has indicated that polysaccharide extracts obtained from *Salmonella* will yield precipitations with homologous antisera. These tests show cross reactions paralleling those of agglutination and conforming to the antigenic classification of *Salmonella* based on the somatic, O antigens (White, 1926; Topley and Wilson, 1936; Kauffmann, 1937). Our interest in examining the antigenic relationship of polysaccharide materials extracted from various *Salmonella* developed from finding a method for obtaining readily the mucoid phase (Beckwith and Morgan, 1937a). This technique was found to be applicable to strains of *Eberthella typhosa* for production of heavily encapsulated organisms (Beckwith and Morgan, 1937b) and thus a higher content of polysaccharide became available.

This study has included an attempt to determine the antigenic relationships of polysaccharides extracted from two strains of *E. typhosa* and one strain each of *Salmonella paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. aertrycke*, *S. enteritidis*, *S. pullorum* and *S. suipestifer*.

MATERIALS AND METHODS

The nine cultures used were smooth stock strains grown on nutrient agar slants and stored in the refrigerator. Before using, biochemical reactions were checked and compared with the reactions as given in Topley and Wilson (1936).

To obtain growth of mucoid character, agar slant cultures were transferred daily three times with incubation at 37°. They, then, were streaked to 1 per cent glucose beef-agar plates, and these were incubated at 16°C. for four days. The mucoid growth was suspended in physiological saline containing 1:10,000 merthiolate.

Antisera for the mucoid antigens were prepared by injecting adult, albino rabbits intravenously at 2-day intervals for a period of six weeks until animals had received a total of 14 cc. of an antigen containing 2×10^9 organisms per cubic centimeter. Blood was obtained one week after the last immunizing dose and the serum prepared.

AGGLUTINATION TESTS

For agglutination, an antigen of organisms in the smooth phase was used, as mucoid organisms are stated by White (1926) to be clumped only with difficulty by the homologous antisera. Cultures of the nine organisms were grown on nutrient agar in Blake bottles for 48 hours at 37°C. and then suspended in physiological saline containing 1:10,000 merthiolate. The suspensions were diluted to uniform turbidity.

One-half cubic centimeter of each of the nine antigens was added to $\frac{1}{2}$ cc. of dilutions of serum which ranged from 1:10 to 1:1280. Readings for complete and partial agglutination were made after two hours in a 37°C. water bath and then overnight in the refrigerator. The observations of these tests are summarized in table 1. The two animals injected with the antigen of *Salmonella pullorum* showed no production of antibodies.

POLYSACCHARIDE EXTRACTION

The method for obtaining the polysaccharide is a modification of the technique of Heidelberger, Kendall and Scherp (1936) who worked with pneumococci. Instead of using 1.2 volumes of 95 per cent alcohol to precipitate the polysaccharide, 4 volumes were employed, as the work of White (1929) indicated that this concentration was necessary in order to precipitate the specific substance of *Salmonella aertrycke*, *S. enteritidis*, *S. suipestifer* and *E. typhosa*.

Mucoid growth of the nine organisms was suspended in physiological saline containing 1:10,000 merthiolate and poured into a flask containing glass beads. The suspension remained in an incubator at 56° for 3–6 hours and then was shaken for 1 hour mechanically. The emulsion was centrifugalized at high speed for ½ hour and the precipitate of cells discarded. The supernatant was poured into a large flask. To 50 cc. of this supernatant, 0.5 gram of sodium acetate was added and then 4 volumes of 95 per cent alcohol, slowly and with continuous shak-

TABLE 1

Cross agglutination reactions

ANTIGENS	DILUTION OF ANTISERA								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertryche*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)	1,280	1,280	160*	10	10	10	80	0	0
E. typhosa (T8)	640	1,280	20	10	10	10	80	0	0
S. paratyphi A	40*	20*	1,280	80	0	10	10	0	0
S. paratyphi B	0	0	20	640	10*	80	0	0	0
S. paratyphi C	0	10*	0	0	80	10*	0	80	0
S. aertrycke	0	0	40	160	0	1,280	0	0	0
S. enteritidis	40*	80*	20	0	0	0	160	0	0
S. suipestifer	0	0	0	0	20	0	0	80	0
S. pullorum	80	40	20	0	0	0	80	0	0

Figures represent the highest dilution of serum that gave complete agglutination after standing overnight in the refrigerator.

0 indicates no agglutination obtained.

* Partial agglutination only obtained to this dilution.

ing. The mixture was placed in the refrigerator overnight, after which most of the alcohol was decanted. The suspension was centrifugalized, the supernatant discarded and the precipitate of polysaccharide resuspended in physiological saline. To 50 cc. gray and opalescent suspension of the polysaccharide 2 grams of sodium acetate were added and the fluid was made faintly acid to litmus by glacial acetic. Four volumes of 95 per cent alcohol were added to reprecipitate the polysaccharide. This resuspension and reprecipitation were repeated three times

for purification. The polysaccharide was finally suspended in physiological saline containing 1:10,000 merthiolate.

These colloidal solutions of the polysaccharide complexes gave faintly positive biuret and strongly positive ninhydrin tests which indicate the presence of peptide linkages. Millon and Hopkins-Cole tests were negative. Esbach (picric acid) and Exton (sulpho-salicylic acid) tests for intact protein were negative. The Molisch test for carbohydrate was strongly positive. The iodine test for glycogen was negative. These tests were

TABLE 2

Cross precipitation reactions of polysaccharides

ANTIGENS DILUTED 1:500	ANTISERA DILUTED 1:2.5								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)	+++	++++	+	+	±	−	±	−	−
E. typhosa (T8)	+++	++++	++	+	±	±	++	−	−
S. paratyphi A	+	+	+++	+	−	±	±	−	−
S. paratyphi B	+	+	++	++	−	+++	−	−	−
S. paratyphi C	±	±	−	−	++	−	−	++	−
S. aertrycke	−	−	+	+	−	+++	−	−	−
S. enteritidis	++	+	+	±	−	−	+++	−	−
S. suipestifer	−	−	−	−	++	−	−	+++	−
S. pullorum	+++	+++	+	+	−	−	++	−	−

− to ++++ indicate the degree of precipitation; ± precipitation recorded with aid of hand lens.

carried out on solutions of polysaccharide complexes containing from 4.3 to 13.5 milligrams per cubic centimeter. The intensity of the biuret test varied directly with the concentration of polysaccharide present in the solution.

The amount of polysaccharide in each of the solutions was determined by the weight of the residue when a measured volume was dried for 24 hours at 80–90°C. Dilutions of 1:500, 1:1000 and 1:5000 by weight were prepared. The immune sera were diluted to 1:2.5 with physiological saline. To ½ cc. of the diluted serum, ½ cc. of the various dilutions of antigens were added.

As controls, ½ cc. of the 1:500 antigen was added to ½ cc. of normal rabbit serum and to ½ cc. of saline. The tubes were shaken and placed in a water bath at 37°. Readings for the amount of precipitate formed were made at the end of 2 hours at 37° and after storage overnight in the refrigerator. The amount of precipitation was recorded as ± to ++++. To summarize the results, table 2 records the amount of precipitate in the tube containing the 1:500 dilution of antigen.

The results of the cross agglutination and cross precipitation tests seem to justify the conclusion that the protein-free polysaccharides obtained by the extraction of mucoid cultures of *E. typhosa*, *S. paratyphi A*, *B* and *C*, *S. aertrycke*, *S. enteritidis*, *S. suipestifer* and *S. pullorum* represent a portion at least of the surface antigen of the organisms. The polysaccharide complex seems to contain all of the major factors of the O antigen as the cross reactions obtained in the agglutination tests are confirmed by the precipitation reactions. Both of these series conform to the cross reactions expected on the basis of the White-Kauffmann antigenic classification of *Salmonella* based on the O surface antigens.

EXPERIMENTS USING ANTISERA PREPARED AGAINST POLYSACCHARIDE EXTRACTS

Using various methods for extraction of the polysaccharide complex, Boivin, Mesrobeanu and Mesrobeanu (1933b); Raistrick and Topley (1934), Morgan (1937) and Henderson and Morgan (1938), prepared extracts from *S. aertrycke*, *Shigella dysenteriae* (Shiga) and *E. typhosa* which would produce agglutinins for the cells and precipitins for the polysaccharide complexes when used to immunize animals. These extracts yielded negative outcomes for intact protein but usually gave positive biuret and ninhydrin tests and a strongly positive Molisch reaction. The chemical analyses of Boivin, Mesrobeanu and Mesrobeanu (1933b) and Morgan (1937) indicate that these substances are polysaccharide-lipoid complexes; the polysaccharide is the soluble specific substance which had been isolated in earlier work.

Our material seems to have some of the same characteristics as the antigenic extracts of the above investigators. To determine whether our preparations were antigenic, adult albino rabbits were immunized with each of the nine extracts. Each animal received the same weight which was as follows: intracutaneously, 13.7 mgm.; subcutaneously, 17.5 mgm.; intraperitoneally, 35 mgm. and intravenously, 10 mgm. This totals 76.2 mgm. The earlier injections were administered intracutaneously and subcutaneously because the material was toxic to the animals.[1]

TABLE 3

Cross agglutination reactions using antisera prepared with polysaccharide extracts

ANTIGENS	DILUTION OF ANTISERA								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)	640	640	20*	10*	10*	10*	40	0	40
E. typhosa (T8)	320	640	40*	10*	40*	10*	20	0	20
S. paratyphi A	40*	40*	80	40*	0	0	10	0	10
S. paratyphi B	20*	20*	80*	80	0	40	20*	0	10*
S. paratyphi C	0	0	0	0	160	0	0	80	0
S. aertrycke	0	0	0	10	0	80	0	0	0
S. enteritidis	40*	10	0	0	0	0	160	0	20
S. suipestifer	0	0	0	0	40	0	0	80	0
S. pullorum	160	160	10*	10*	0	10*	80	0	80

Figures in the table represent the highest dilution of serum that gave complete agglutination after standing overnight in the refrigerator.

* Partial agglutination only obtained to this dilution.

0 indicates no agglutination obtained.

A week following the final immunizing dose, the animals were bled and serum prepared. All treated animals showed production of agglutinins and precipitins.

AGGLUTINATION TESTS

Agglutination tests were made using the technique outlined previously. Table 3 summarizes the results.

[1] Two adult albino rabbits receiving 2 mgm. of extract of *E. typhosa* intravenously died within 3 hours.

PRECIPITATION TESTS

Cross precipitation tests were set up as before using the antisera prepared with the polysaccharide extracts. The precipitations are summarized in table 4.

The precipitation tests give striking confirmation of the cross reactions obtained by agglutination. The antisera prepared with the antigenic polysaccharide extracts give the same cross reactions as those obtained with antibacterial sera. The extract therefore must contain the O antigens of the cells.

TABLE 4

Cross precipitation reactions using antisera prepared with polysaccharide extracts

ANTIGENS DILUTED 1:500	ANTISERA DILUTED 1:2.5								
	E. typhosa (T1)	*E. typhosa* (T8)	*S. para A*	*S. para B*	*S. para C*	*S. aertrycke*	*S. enteritidis*	*S. suipestifer*	*S. pullorum*
E. typhosa (T1)	++++	+++	±	+	±	±	++	−	+++
E. typhosa (T8)	+++	++++	±	+	−	+	++	−	+++
S. paratyphi A	+	+	++	+	−	+	−	−	+
S. paratyphi B	+	±	+	++	+	++	−	−	−
S. paratyphi C	±	−	−	−	+++	−	−	++	−
S. aertrycke	−	−	−	++	−	+++	−	−	−
S. enteritidis	++	+++	−	±	−	−	+++	−	+
S. suipestifer	−	−	−	−	+++	±	−	+++	−
S. pullorum	++	+++	±	±	−	+	++	±	++++

− to ++++ indicate degree of precipitation after standing overnight in refrigerator; ± precipitation recorded with aid of hand lens.

SKIN REACTIONS

Animals immunized with bacterial antigens have been found to show skin sensitivity to homologous polysaccharides. Zinsser and Parker (1923) noted that animals immunized with intracutaneous injections of *E. typhosa* presented cutaneous reactions after the injection of an extract of *E. typhosa*. This material was precipitated by alcohol and gave negative biuret, Millon Hopkins-Cole and sulpho-salicylic acid tests for protein. Francis and Tillet (1931) found that animals immunized against pneu-

mococci demonstrated type-specific cutaneous reactions when the specific capsular polysaccharides were injected.

Each of the animals immunized with bacterial antigens received intracutaneously 0.3 cc. of a preparation containing 5 mgm. of the polysaccharide complex per cubic centimeter. Reactions were read at 24 and 48 hours. The results were not in accord with previous immunological observations of similar nature because the non-immunized controls likewise showed cutaneous responses to the injections. This response usually consisted of an erythematous area of $\frac{1}{2}$ to $1\frac{1}{2}$ cm. in diameter with a raised center. One to two weeks after the injection, a round, firm lump $\frac{1}{2}$ cm. in diameter usually formed at the site of injection. This was filled with a white exudate of the consistency of lard surrounded by a well defined wall of fibrous tissue. There was no indication of infection. These sterile abscesses occurred in animals both immunized and untreated.

DISCUSSION

The cross reactions obtained by use of agglutination and precipitation with the anti-bacterial and anti-polysaccharide complex antisera are in accord with the antigenic classification of White (1926), Topley and Wilson (1936) and Kauffmann (1937). The immunological relationships given by *E. typhosa*, *S. enteritidis* and *S. pullorum* would be expected as they share the O antigen IX and the accessory factor XII. Cross reactions in precipitation tests between *E. typhosa* and *S. enteritidis* were obtained also by Furth and Landsteiner (1928), (1929) using anti-bacterial sera and non-antigenic polysaccharide extracts. The cross reactions between *E. typhosa* and *S. paratyphi B* are explained by the common content of the accessory O antigen XII. Furth and Landsteiner (1928), (1929) noted this relationship. Common precipitation and agglutination of *E. typhosa* and *S. paratyphi A* is not accounted for by the presence of a common identified O antigen.

The marked antigenic relationship between *S. paratyphi B* and *S. aertrycke* as indicated by the agglutination and precipitation tests is in conformity with their common possession of

the O antigens I, IV, V and the accessory factor XII. This relationship is reported by Raistrick and Topley (1934) and Boivin and Mesrobeanu (1937) using their antigenic polysaccharide complexes and antisera prepared with them for precipitation tests. Antigens VI and VII shared by *S. paratyphi C* and *S. suipestifer* account for their close antigenic relationship.

The immunizing activity of the polysaccharide extract prepared indicates that it contains all of the factors of the O antigen of the organisms identified in the White-Kauffmann scheme of classification. It is antigenic though free from demonstrable intact protein. Its properties indicate that it is similar or identical to the antigenic extracts prepared by Boivin, Mesrobeanu and Mesrobeanu (1933b), Raistrick and Topley (1934) and Morgan (1937), which are probably polysaccharide-lipoid complexes.

The tendency of the polysaccharide extract on intracutaneous injection in rabbits to form sterile abscesses is of interest. Obviously this action is not related to sensitivity as it occurs in normal as well as in immunized animals. The response which forms a sterile abscess indicates some peculiar property of the extract.

The polysaccharide extract may contain inert materials since White (1929) found that precipitation of the polysaccharide of *S. aertrycke* with 2 volumes of alcohol produced material serologically inert while 4 volumes caused the specific substances to precipitate. However, in the precipitation tests with our polysaccharide extract, all of the material was thrown down by the immune sera for the supernatant was clear.

The authors are indebted to Mr. H. C. Upham for technical aid in this work.

SUMMARY

1. An extract of polysaccharide prepared from mucoid cultures of *Eberthella typhosa, Salmonella paratyphi A, Salmonella paratyphi B, Salmonella paratyphi C, Salmonella aertrycke, Salmonella enteritidis, Salmonella suipestifer* and *Salmonella pullorum* gave cross precipitation tests which parallel the cross agglutination

reactions obtained by use of antibacterial sera for these organisms. These cross reactions conform to the antigenic classification of White and of Kauffmann.

2. These polysaccharide extracts free of intact protein give rise to the formation of precipitins for the polysaccharides and agglutinins for the organisms when used to immunize rabbits.

3. The antisera prepared against the polysaccharide extracts show all of the cross reactions of the antibacterial sera in tests by agglutination and precipitation.

4. The intracutaneous injection of the polysaccharide in normal or immune rabbits leads to the formation of sterile abscesses.

REFERENCES

BECKWITH, T. D., AND MORGAN, H. R. 1937a Production of mucoid dissociants of *B. coli* by thermal variation. Proc. Soc. Exper. Biol. and Med., **36**, 606–607.

BECKWITH, T. D., AND MORGAN, H. R. 1937b A heavily encapsulated form of *B. typhosus*. Proc. Soc. Exper. Biol. and Med., **37**, 682–683.

BOIVIN, A., MESROBEANU, I., AND MESROBEANU, L. 1933a Technique pour la preparation des polysaccharides microbiens specifiques. Comp. Rend. Soc. de Biol., **113**, 490–492.

BOIVIN, A., MESROBEANU, I., AND MESROBEANU, L. 1933b Extraction d'un complexe toxique et antigenique à partir du Bacille d'Aertrycke. Comp. Rend. Soc. de Biol., **114**, 307–310.

BOIVIN, A., AND MESROBEANU, L. 1937 Sur le pouvoir protecteur antitoxique des serums obtenus en injectant a l'animal des endotoxines glucido-lipidiques. Comp. Rend. Soc. de Biol., **124**, 1092–1094.

FRANCIS, T., AND TILLETT, W. S. 1931 Cutaneous reactions in rabbits to the type-specific capsular polysaccharides of pneumococcus. J. Exp. Med., **54**, 587–595.

FURTH, J., AND LANDSTEINER, K. 1928 On precipitable substances derived from *B. typhosus* and *B. paratyphosus B*. J. Exp. Med., **47**, 171–183.

FURTH, J., AND LANDSTEINER, K. 1929 Studies on the precipitable substances of bacilli of the Salmonella group. J. Exp. Med., **49**, 727–743.

HEIDELBERGER, M., KENDALL, F. E., AND SCHERP, H. W. 1936 The specific polysaccharides of types I, II and III pneumococcus. A revision of methods and data. J. Exp. Med. **64**, 559–572.

HENDERSON, D. W., AND MORGAN, W. T. J. 1938 The isolation of antigenic substances from strains of *Bact. typhosum*. Brit. J. Exper. Path., **19**, 82–94.

KAUFFMANN, F. 1937 Salmonella-Probleme. Ztschr. f. Hyg. u. Infectionskr., **120**, 177–197.

MORGAN, W. T. J. 1937 Studies in immuno-chemistry. II. The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). Biochem. J., **31**, 2003–2021.

RAISTRICK, H., AND TOPLEY, W. W. C. 1934 Immunizing fractions isolated from *B. aertrycke*. Brit. J. Exper. Path. **15**, 113–129.

TOPLEY, W. W. C., AND WILSON, G. S. 1936 The Principles of Bacteriology and Immunity. Second edition, 550–553. Wm. Wood and Co., Baltimore.

WHITE, P. B. 1926. Further studies of the Salmonella group. Brit. Med. Res. Council Special Report Series, No. 103.

WHITE, P. B. 1929 Notes on intestinal bacilli with special reference to S and R races. Method for preparing the soluble specific substances. J. Path. and Bact., **32**, 85–94.

ZINSSER, H., AND PARKER, J. T. 1923 Further studies on bacterial hypersusceptibility. J. Exp. Med., **37**, 275–302.

MENINGITIS CAUSED BY ATYPICAL GRAM-NEGATIVE COCCI

HOBART A. REIMANN, AND RUDOLPH W. KOUCKY

Department of Medicine of the Jefferson Medical College and Hospital and the Pathological Service of the University of Minnesota Hospital

Received for publication September 28, 1938

Gram-negative cocci found in samples of purulent spinal fluid during routine laboratory examinations are apt to be reported as meningococci without further confirmation. While the meningococcus no doubt is the most common gram-negative coccus associated with acute meningitis, there are many others which play a rôle, particularly in sporadic cases. Error in diagnosis is also apt to occur in certain cases of meningitis caused by metachromatic cocci or by gram-positive cocci if the Gram stain is improperly applied, and also as a result of the fact that cocci, ordinarily gram-positive, may be decolorized when they are dead or phagocyted. Recognition of these problems and greater care in laboratory diagnosis will prevent wasting antimeningococcus serum in disease not caused by typical meningococci and may help to explain the failure of specific antimeningococcus serum in certain instances.

Branham, Mitchell and Brainin, (1938) recently pointed out the difficulties attending the differentiation of even such well-known bacteria as gonococci from the meningococcus in cases of meningitis. Many of the strains of gonococci which they studied were agglutinated by polyvalent antimeningococcus serum. Sugar fermentation reactions were somewhat more reliable in differentiation but the most fundamental differences appeared in cultural studies pertaining to colony morphology and growth requirements. Even more confusion in diagnosis is apt to arise in dealing with other gram-negative cocci such as *Micrococcus catarrhalis, Micrococcus florens, Micrococcus crassus,*

Micrococcus flavus and other still unnamed varieties, or when multiple infection with two or more strains occurs. German bacteriologists thirty or forty years ago were especially concerned with the problem and many of their publications are referred to in the papers of Forbes (1920) and others. A prolonged controversy arose as to whether the different atypical microörganisms encountered were (a) separate and distinct types of bacteria, (b) variant or mutant forms of the meningococcus, (c) secondary invaders, or (d) contaminants. The problem is illustrated in the following two case reports and studies, the first of which apparently involved an atypical gram-negative coccus; and the second, a type I meningococcus with unusual growth characteristics, together with an atypical staphylococcus.

Accurate knowledge of a complex problem is always desirable.

Case 1 (by courtesy of Dr. I. McQuarrie). A girl, aged 7, suddenly vomited and had diarrhea and headache. She was admitted to the pediatric department of the University of Minnesota Hospital four days later with a diagnosis of meningitis. Rigidity of the neck, Kernig's sign and other disturbed reflexes were noted. A blood culture was negative. The spinal fluid was under normal pressure, but was turbid, contained 68 leukocytes per cu. mm. (56 per cent polymorphonuclear cells) and numerous extracellular and intracellular gram-negative diplococci, which were reported to be meningococci. Antimeningococcus serum was promptly injected intraspinously and subsequent doses were given daily for 6 days. The child improved and her temperature returned to normal about 10 days after admission to the hospital.

Bacteriologic studies. Gram-negative cocci were seen in smears from the spinal fluid before serum injection and on the day after. They were cultivated from the same samples of fluid and also from spinal fluid taken on the fourth day of treatment. Growth was obtained from the spinal fluid only in liver-peptone-broth tubes. The broth became turbid after 24 hours at 37°C. and no sediment or pellicle formed. Subcultures grew on ordinary laboratory media at temperatures between 10° and 37°C., better toward the higher level. On plain agar and blood agar plates the colonies after 24 hours at 25°C. were about a millimeter

in diameter, dull, grayish and translucent. Several days later, the colonies measured 3 or 4 millimeters in diameter and opaque yellowish-gray sectors and wedges formed in many of the colonies. Subcultures of the translucent portions reproduced translucent colonies with later opaque sector development. Subcultures of the opaque area produced similar opaque colonies (fig. 1) in which translucent sectors and wedges appeared after a few days. In one opaque point colony a large translucent wedge formed and at the edge of the translucent portion an opaque wedge developed. The cocci from both forms were identical in morphology and in biological behavior. They were usually

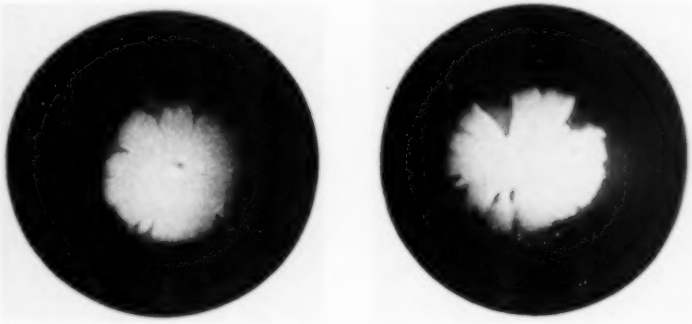


FIG. 1. COLONIES ABOUT TWO WEEKS OLD FROM CULTURE OBTAINED IN CASE 1

Magnified about × 6. Several translucent sectors are shown in the opaque colonies.

gram-negative but, unless thoroughly decolorized, many retained the purple color, especially in old cultures. The cocci were oval or spherical, usually in pairs with adjacent sides flattened, occasionally single or in clusters. The long axis of the diploforms was at right angle to the line of union. There was marked variation in size. Cocci from both opaque and translucent colonies caused hemolysis on blood agar, formed an acid coagulum in milk after 7 days at 37°C, slowly liquefied gelatin, caused no change in maltose, mannitol, or sucrose broth, but formed acid after 7 days in glucose and less in lactose broth. No agglutination occurred in various types of antimeningococcus serum

obtained from Dr. C. P. Miller of Chicago, from Dr. S. Branham of Washington and from commercial sources. Broth cultures were avirulent when injected intraperitoneally into white mice, guinea pigs and rabbits.

It is obvious that the bacterium is not a meningococcus nor does its behavior coincide in all respects with the criteria laid down for *Micrococcus catarrhalis*, *Micrococcus florens* (Davison, Davison, and Miller, 1917), *Micrococcus crassus* (Elser and Huntoon, 1909), *Micrococcus flavus* and others (Branham, 1930). It has many, but not all of the characteristics of each of the varieties named, but for reasons given in the discussion it does not seem desirable to give the strain a new name.

The second case apparently involves the problem of dual infection and of type transformation which confronted Von Hibler (1907), Stoevesandt (1908), Forbes (1920), Köhlisch, (1915), McDonald (1908), Kempf, Gilman and Zerfas (1933) and others (Sen, 1936).

Case 2. A young man, age 23, was said to have had meningitis following sinusitis in 1932. On April 10, 1936 he noted sore throat and awakened the next day with severe headache followed by vomiting and chills. He was then admitted to the Student Health Service of the University of Minnesota Hospital. His throat was inflamed and a shallow ulcer was seen in the right tonsillar fossa. His neck was slightly rigid; there was general hyperesthesia of the skin, but no disturbance of reflexes until later in the day when Kernig's sign appeared. The leukocytes numbered 27,000 and a gram-positive coccus was cultivated from the blood. The spinal fluid was under 24 mm. of mercury pressure and contained 8000 cells of which 70 per cent were polymorphonuclear. A direct smear of the spinal fluid examined in the Minnesota State Board of Health laboratory showed numerous intracellular gram-negative diplococci. The patient was then given antimeningococcus serum intraspinally and intramuscularly. There was considerable improvement in his condition on the following day, but the neck was still rigid. His temperature declined and became normal after several days.

Bacteriologic studies. As a part of a routine procedure, portions of the same specimen of spinal fluid were sent to the Min-

nesota State Board of Health laboratory and to the University Hospital laboratory. The former reported the presence and subsequent cultivation of typical meningococci and the hospital laboratory reported the presence of gram-positive intracellular diplococci. The gram stain of the latter was retained even when decolorization was carried to the point at which all color was gone from the nuclei of the leukocytes in the smear.

The meningococcus. Because of the different reports from the two laboratories, cultures of the meningococcus first isolated were sent to Dr. C. P. Miller and to Dr. Sara Branham both of whom confirmed its identity as type I meningococcus. The strain was subsequently tested in our laboratory and found to have certain unusual characteristics. The culture from a Loeffler medium slant was seeded in 9 stab punctures in a deep (10 mm. thick) plain agar plate. After 48 hours at room temperature growth appeared at 3 of the 9 points of inoculation. The colonies were translucent and composed mostly of gram-negative diplococci, but, here and there, were found distinctly gram-positive cocci, even after thorough decolorization. Another culture was made in a 100 cc. flask of broth and incubated 6 days at 37°C. Subcultures on plain agar showed two forms of colony, large and small, composed of similar cocci most of which were gram-negative, but a few were gram-positive. Numerous tetrads were present and the cocci were pleomorphic and metachromatic.

The Gram-positive cocci isolated in the hospital laboratory. Gram-positive cocci, which were cultivated from the spinal fluid and from the blood before serum treatment, and from the spinal fluid on the day after, were apparently identical. Subsequent cultures from the blood and spinal fluid were sterile. Fluid from the second spinal fluid specimen was inoculated in numerous stabs in a plain agar plate and growth occurred at each site, the colonies measuring 2 mm. after 6 days at 25°C.

A culture in liver-peptone-broth from the first specimen was plated on plain agar. Three forms of colonies appeared (1) many small translucent ones, (2) several larger, thicker, opaque brownish ones and (3) a few opaque white ones. The appearance

of a variety of colonies was similar to the experience reported in a case of meningitis caused by *Micrococcus tetragenus*, and was regarded as evidence of variation in a single strain of bacteria. Subcultures from each of the colony types bred true. The brown colonies on occasion developed both translucent and white forms as wedges or daughter colonies. The cocci isolated from the blood first produced colonies of the white type, and in later subcultures similar translucent, and the brownish, forms appeared.

Cocci from all three colony types were gram-positive and were arranged singly, in pairs, in tetrads and in clusters like staphylococci. Certain characteristics of the three types are shown in table 1.

TABLE 1

Maltose mannitol lactose glucose sucrose milk gelatin hemolysis

White	++	−	+	+	+	Ft. acid	Liquefied	±
Brown	+	−	+	+	+	Acid	Liquefied	+
Translucent	+	+	+	+	+	Thick coagulum	Liquefied	++

Tests made by Dr. W. A. Kreidler.

Cocci from all three forms were avirulent for white mice after intraperitoneal inoculation. Agglutination tests were negative in serum obtained from the patient, but were irregularly positive in low dilutions of several samples of antimeningococcus serum, regardless of source or type.

We believe that the patient had primary meningococcus meningitis and was host to a staphylococcus which became a secondary invader. The three colony forms were regarded as variants of the strain of staphylococcus in question. It is too remote a possibility to suggest that the staphylococcus was a mutant form of the meningococcus. A few experiments were made to determine whether or not growth of meningococci in the liver-peptone-broth used in our laboratory tended to transform them into gram-positive cocci. Nine strains of meningococci were grown for various periods up to 6 months in liver-peptone-broth. Transplants were made once or twice a week.

Plate cultures from these tubes at various times revealed the usual type of colony which was smooth and transparent, and two variant forms, one, rough and the other, moist and sticky. No gram-postive cocci were encountered from any of the colonies examined.

It is probable that under ordinary routine diagnostic conditions the gram-positive cocci isolated from the patient's spinal fluid in our laboratory would be disregarded or dismissed as contaminants, yet the presence of identical cocci in the blood and similar experiences of other observers, notably Köhlisch (1915) and Kempf and his associates (1933), show that the occurrence of more than one species of bacteria in cases of meningitis is not uncommon.

DISCUSSION

The problem in both cases appears to involve the question of bacterial variation or type transformation as described previously (Reimann, 1935) in a case of meningitis caused by *Micrococcus tetragenus* in which three colony forms of *Micrococcus tetragenus* were immediately detected and later 13 additional variant forms of the original strain were isolated. These various types derived from each other differed considerably in several of the biological tests commonly used. Certain of the differences were as great or greater than those which are customarily used to separate and identify the closely related strains of the *Neisseria* group and the various atypical meningococcus strains (Reimann, 1937). In case 1, the gram-negative bacterium showed two colony forms neither of which had characteristics to identify it with previously established varieties. It was not determined however, whether they were variant forms or types of certain known varieties or a separate variety. It is obvious, however, that not all gram-negative cocci found in the spinal fluid in cases of meningitis are meningococci.

The problem in case 2 was still more complicated. The type I meningococcus recovered grew on plain agar at room temperature, which is at variance with all but a few descriptions of meningococci, and further, many of the cocci retained the

gram stain. The latter observation was similar to that of McDonald (1908) and others, yet Murray (1929) voices the general opinion that meningococci are never gram-positive. Two colony types were observed in old cultures as noted also by McDonald (1908) and Canti (1918).

The gram-positive cocci isolated from the spinal fluid and from the blood, might have been looked upon either as mutation forms of the meningococcus as suggested by Köhlisch (1915), or as staphylococci and secondary invaders. Here again evidence of type transformation was present among the three colony forms derived. There were therefore obtained from the same spinal fluid sample five different colony types, namely, two forms of the meningococcus and three of the staphylococcus. Under the old monomorphic conception of classification these may have been mistaken for five separate strains, or as evidence of contamination, but in reality they represent the variant forms or types of a member of the *Neisseria* group and those of a staphylococcus which we believe was a secondary invader. Similar circumstances may have caused the polemic of thirty years ago about the relationship between the meningococcus, the *Diplococcus crassus* of Jaeger and Von Lingelsheim, and the staphylococcus. Viewed in the light of present knowledge of bacterial variation and multiple infection, the problem still seems complicated but less obscure. On the evidence submitted, one may suggest the following possibilities: first, it would seem that any of a number of bacterial strains ordinarily harbored in the nasopharynx or elsewhere may become invasive and cause meningitis when the resistance of the host is reduced; second, a given strain may be present in several variant forms of type or culture phase (mucoid, smooth and rough), and third, secondary infection with another organism with its variant forms may also be present. This view of the matter reconciles many of the old ideas since it is now known that numerous bacteria may cause meningitis and any given strain may manifest itself in a number of variant forms.

One is inclined to doubt with Hadley (1927) and Sherman (1937) whether it is possible ever to classify bacteria except on

a broad basis. Sugar fermentation, agglutination and other tests have lost much of the importance heretofore attached to them in differentiation. In our own experience many of these tests give inconstant results in the hands of different workers and even for the same person working with the same materials and same technic at different times. Certain types of bacteria indeed seem to have relatively constant characteristics, but the evidence of type and culture phase instability as demonstrated among pneumococci, *Micrococcus tetragenus* and staphylococci suggests that similar variation may occur among other bacterial species. Evidence of type variation among meningococci has already been published (Atkin, 1925; Branham, 1937).

SUMMARY

Two cases of meningitis associated with atypical bacteria were studied. From the first patient (case 1), a gram-negative diplococcus with characteristics different from other classified strains was isolated. The bacterium gave rise to two interchangeable colony forms. From the second case, two species of cocci were obtained; one was a type I meningococcus which had the unusual ability to grow on plain agar at room temperature and had gram-positive elements, the other was a staphylococcus which was represented by three interchangeable colony forms.

These studies suggest that under ordinary laboratory routine examination, atypical colonies may appear on culture plates. Such colonies are usually ignored or dismissed as contaminants, but they may actually be part of the pattern of variation of the bacterium concerned. Recognition of the phenomenon of bacterial variation and type transformation would remove much confusion and would probably reduce the number of separate varieties of bacteria now believed to exist.

REFERENCES

ATKIN, E. E. 1925 Significance of serological types of gonococcus. Brit. J. Exp. Path., **6**, 235-246.

BRANHAM, S. E. 1930 A new meningococcus-like organism. (*Neisseria flavescens* N.S.) from epidemic meningitis. Public Health Reports, **45**, 845-849.

BRANHAM, S. E. 1937 The significance of serologic types among meningococci. J. A. M. A., **108**, 692–696.

BRANHAM, S. E., MITCHELL, R. H., AND BRAININ, W. 1938 Gonococcic meningitis. J. A. M. A., **110**, 1804–1806.

CANTI, R. G. 1918 An anomalous meningococcus. J. Hyg., **17**, 281–289.

DAVISON, W. C., DAVISON, A. S., AND MILLER, M. K. 1917 Meningitis in an infant due to a hitherto undescribed organism, *Micrococcus florens*. J. Exp. Med., **26**, 779–793.

ELSER, W. J., AND HUNTOON, F. M. 1909 Studies on meningitis. J. Med. Res., **20**, 377–451.

FORBES, J. G. 1920 *Diplococcus crassus* and meningitis. Lancet, **2**, 690–692.

HADLEY, P. 1927 Microbic dissociation. J. Inf. Dis., **40**, 1-312.

KEMPF, G. F., GILMAN, L. H., AND ZERFAS, L. G. 1933 Meningococcic meningitis and epidemic meningo-encephalopathy. Arch. Neur. and Psych., **29**, 433–453.

KÖHLISCH 1915 Bakteriologische Befunde bei einem Fall vom Meningokokkensepsis; gibt es eine Mutation bei Meningokokken? Zeit. f. Hyg., **80**, 404–430.

MCDONALD, S. 1908 Observations on epidemic cerebro-spinal meningitis. J. Path. and Bact., **12**, 442–454.

MURRAY, E. G. D. 1929 The meningococcus. Med. Res. Council, Spec. Rep. No. 124. London.

REIMANN, H. A. 1935 *Micrococcus tetragenus* infection. I. J. Clin. Invest., **14**, 807–814.

REIMANN, H. A. 1937 Bacterial type transformation. J. Bact., **33**, 499–511.

SEN, B. B. 1936 An atypical meningococcus isolated from cerebrospinal fluid in the Calcutta meningitis epidemic of 1934–35. J. Bact., **32**, 293–297.

SHERMAN, J. M. 1937 The streptococci. Bact. Rev., **1**, 3–97.

STOEVESANDT, K. 1908 Erfahrungen bei der Bakteriologischen Untersuchung Meningitisverdächtigen Materials. Zentralbl. f. Bakt., **46**, 295–309.

VON HIBLER, E. 1907 Bakteriologischer Bericht über drei Fälle von Cerebrospinal meningitis. Wien. Klin. Wochsch., **20**, 961–967.

AN IMPROVED METHOD FOR TESTING ANTISEPTIC DUSTING POWDERS

JOHN H. BREWER

Bacteriological Laboratory, Hynson, Westcott and Dunning Company, Baltimore, Maryland

Received for publication October 6, 1938

As Garrod (1935) and others have pointed out, there is a definite need to test antiseptics by methods which more closely approximate the actual conditions of use rather than by arbitrary laboratory procedures.

The agar-plate method suggested by Circular 198, United States Department of Agriculture, for testing dusting powders is a very convenient method but should be modified to prevent misleading results and to simulate more closely actual use.

In some recent work with antiseptic dusting powders, it was found that when powders containing some of the essential oils, known to be only slightly bacteriostatic, were placed on agar plates in the amounts shown in the U. S. D. A. Circular 198, a good zone of inhibition would be produced. When amounts actually used in practice were tried, no zone of inhibition was obtained. It was then decided to modify the test in such a way that the amount of dusting powder would be more nearly equivalent to the amount which actually adheres to the skin.

The method found to be most satisfactory is as follows:

Several circular shields are cut from 3 x 5 filing cards. These shields have a 10 mm. square cut from the center and a small tab to facilitate handling. About twenty-five of these shields are placed in a petri dish and sterilized in the hot air oven. The agar plates are made and seeded with *Staphylococcus aureus*, as suggested in the U. S. D. A. circular (0.1 cc. of a twenty-four hour broth culture to 20 cc. of melted agar previously cooled to 42° to 45°C.). Unglazed porcelain-top dishes are used so that

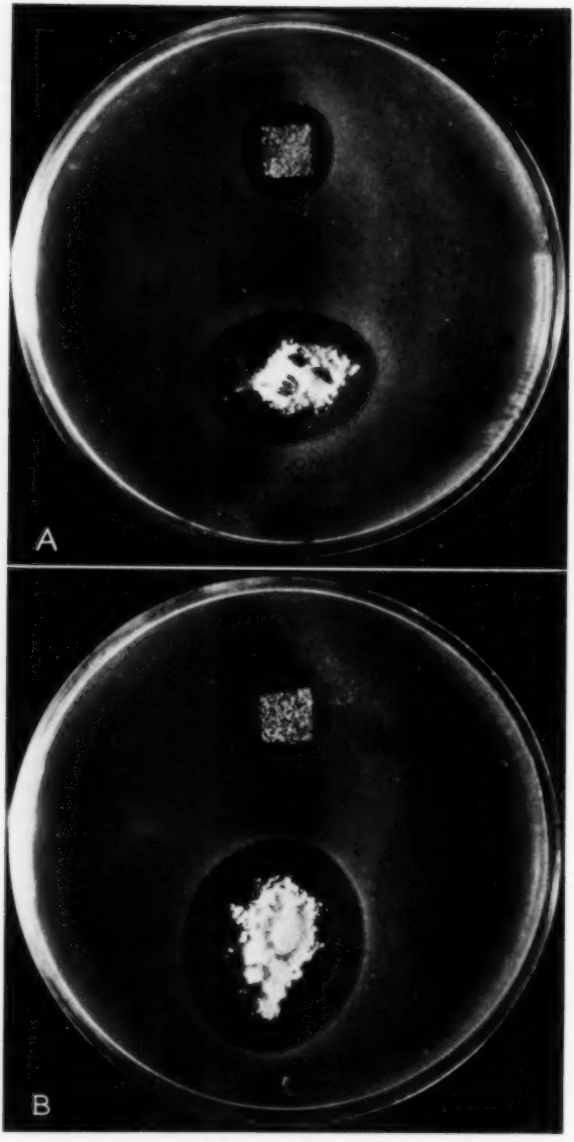


FIG. 1. *A*, plates showing inhibition with small amounts of powder which will adhere to skin; *B*, plates showing inhibition when large amounts are used, but no bacteriostatic action when amounts equivalent to those actually employed on the skin are tested.

there will be no collection of moisture on the surface of the medium. After the agar has hardened, one of the sterile shields is lifted onto the surface by means of the tab which is bent so that it does not touch the agar. About a gram of the dusting powder to be tested is placed on a piece of sterile gauze, approximately 4 inches square (52 mesh Brookside bunting was used), and the corners brought together to form a small bag. The powder is dusted from this bag onto the shield until the 10 mm. square of agar is just covered. The mask is removed, placed in a discard jar, and the petri dish turned upside down to let any excess powder fall off. The lid is replaced and the plate incubated. If desirable at this point, a small spatula of the dusting powder may be placed on the agar opposite the 10 mm. square in order to compare the results with the U. S. D. A. method.

When three of the most popular antiseptic dusting powders on the market and that prepared according to the formula given in the National Formulary for an antiseptic dusting powder were tested by this method, the N. F. powder and only one other gave zones of inhibition when amounts equivalent to those which will adhere to the skin were tested (fig. 1, *A*). Two of the most popular brands which give good zones of inhibition by the U. S. D. A. method give no zones at all when amounts actually used are tested (fig. 1, *B*).

SUMMARY

A simple method of testing antiseptic dusting powders which more closely simulates the actual conditions of use has been described. This test requires the additional use of masks made from 3 x 5 filing cards and a small piece of fine mesh gauze.

This test eliminates false results due to concentration of essential oils, standardizes the zone of application of the dusting powder, and thereby unifies the results for comparison.

REFERENCES

GARROD, LAWRENCE P. 1935 The Laboratory Testing of Disinfectants. British Medical Journal, **1**, 5-8.

RÜEHLE, G. L. A., AND BREWER, C. M. 1931 Methods of Testing Antiseptics and Disinfectants, Circular 198, U. S. Department of Agriculture.

THE PATTERN OF DISSOCIATION IN HEMOPHILUS INFLUENZAE

CAROLINE A. CHANDLER, LEROY D. FOTHERGILL AND JOHN H. DINGLE

WITH THE TECHNICAL ASSITANCE OF MARGARET S. MCMILLAN

Department of Bacteriology and Immunology, Harvard University Medical School

Received for publication October 8, 1938

In recent communications, Hadley (1937), Dawson (1934) and others have pointed out that most, if not all micro-organisms exhibit a similar dissociative pattern. Dawson (1934, 1938), in studies on the pneumococci and the hemolytic streptococci in particular, has shown that the pattern of variation in these two organisms is strikingly similar. In view of this, both Dawson and Hadley have proposed that a uniform nomenclature for all micro-organisms be adopted to designate the three chief phases of dissociation—i.e., mucoid (M), smooth (S) and rough (R).

In 1931, Pittman described a smooth and a rough form for *Hemophilus influenzae*. Smooth strains were characterized by (a) the appearance of their colonies, which were large, smooth, mucoid, opaque and iridescent in obliquely transmitted light; (b) the fact that the bacteria were capsulated; (c) the production of a soluble specific substance (present in culture filtrates and washings of the bacteria). Rough strains formed colonies which were rough and irregular in outline, smaller in size than the S colonies, less opaque and non-iridescent. The organisms were not capsulated, and none of the R strains produced soluble specific substance. The S strains were found to be more pathogenic for laboratory animals than the R strains.

Huntington (1935), referring to Dawson's classification of the pneumococci, suggested that the same terminology be adopted to designate the dissociative pattern of *H. influenzae*. Accord-

ingly, he applied the term "mucoid" to the strains which produced mucoid, fluorescent colonies, capsulated organisms and soluble specific substance (i.e., Pittman's S). He went no further, however, than stating that the S form was the "ordinary" strain and the R the rough variant.

In previous reports by the authors (Chandler, Fothergill and Dingle, 1937; Fothergill and Chandler, 1936; Fothergill, Dingle and Chandler, 1937) the problem of variation in *H. influenzae* has been discussed, particularly from the point of view of its relationship to morphologic, serologic and pathogenic characteristics of the organism. The terminology previously employed by them to describe the dissociative phases of this organism has not, unfortunately, been of aid in clarifying the confused nomenclature. In the present paper, the problem of variation in *H. influenzae* is reviewed in the light of recent observations on microbic dissociation. The data obtained indicate that the three-phase system of dissociation holds for this organism.

The following section headed *Experimental* is a compilation of the authors' earlier observations, together with additional experimental data hitherto unpublished.

EXPERIMENTAL

Methods

Media: broth. To provide the necessary growth factors for this organism, Filde's peptic digest-blood was added to ordinary infusion broth to a concentration of 5 per cent.

Plates. In order to study colonial morphology and variation, a transparent agar medium was used similar to that described by Levinthal and Fernbach (1922). Streak plates were invariably inoculated from broth cultures in such dilution as to give widely separated colonies. A Zeiss colony microscope was employed in studying colonial morphology. The presence or absence of fluorescence was determined by examining the plates in transmitted, artificial light.

Capsule stain. Several capsule stains were employed, including Hiss', Wright's and Enders'.

Precipitin tests. Precipitin tests were performed according to the technique described in a previous paper (1936).

Virulence determinations. Virulence determinations in mice were

carried out by the mucin method (Fothergill, Dingle and Chandler, 1937a). Henceforth, in this paper, the term "mouse-virulence" implies its determination by this technique.

Cultures. All strains employed were originally isolated from (a) the spinal fluid of patients with meningitis or the blood of patients with bacteremia and (b) the respiratory tract of patients with mild upper respiratory infections. All the rough strains referred to in this paper were derived from Type *b* mucoid strains by prolonged growth on artificial media or repeated transfer in Type *b* immune serum broth.

Characteristics of strains isolated from the spinal fluid of patients with H. influenzae meningitis and the blood of patients with H. influenzae bacteremia

Colonial morphology. Large, mucoid, smooth, convex, glistening, opaque, markedly fluorescent in transmitted light.

Morphology of organisms. Capsulated, coccobacilli or short rods, uniform in size and staining properties.[1]

Growth in broth. Diffuse.

Type-specificity. An essentially homogeneous group, serologically, producing soluble specific substance.

Virulence. Virulent for man (probably for young children and infants only (Fothergill and Wright, 1933)). Virulent for mice by mucin technique.

Stability. Very unstable on primary isolation. Plates of first subculture frequently show, in addition to the mucoid colonies, a few variants of the rough type. Once growth is established in a favorable medium, however, strains become very stable and produce only mucoid, fluorescent colonies. After varying lengths of time (6 months to 2 years) variants begin to appear.

Characteristics of strains isolated from the upper respiratory tract in non-specific respiratory infections

A. Predominant strains

Colonial morphology. Small, smooth, convex, slightly conical, bluish, translucent, non-fluorescent in transmitted light.

[1] Smears made directly from the spinal fluid frequently showed very pleomorphic forms, but these were replaced by non-pleomorphic coccobacilli after subculture.

Morphology of organisms. Non-capsulated coccobacilli or short rods, uniform in size and staining properties; larger rods occasionally present.

Growth in broth. Diffuse.

Type specificity. Serologically heterogenous; specific soluble substance not present.

Virulence. May or may not be virulent for man; slightly virulent to avirulent for mice by mucin technique.

Stability. Relatively stable when freshly isolated, but some strains after prolonged growth on artificial media produce colonies which are rough. Spontaneous reversion to the mucoid form has not been observed.

B. Other strains

Approximately 80 to 90 per cent of strains isolated from the respiratory tract in mild respiratory infections exhibit the characteristics listed above. Aside from occasional "*intermediate*" forms, the rest of the strains isolated from this source fall into (a) the mucoid group and are fluorescent, capsulated and type-specific (a, b, c, d, e, or f) (Pittman, 1931; Platt, 1937), (b) the rough group and are non-fluorescent, non-capsulated, non-type-specific and avirulent for mice.

Characteristics of strains dissociated by means of prolonged growth on artificial media or by repeated transfer in broth containing type specific immune serum

Colonial morphology. Fairly large, rough, slightly convex to flat, edge irregular to serrated, translucent or slightly opaque, bluish, non-fluorescent in transmitted light.

Morphology of organisms. Non-capsulated, long rods, clubs, filaments, very irregular in size, shape and staining; markedly pleomorphic.

Growth in broth. Granular or flocculent.

Type specificity. Serologically heterogenous; specific soluble substance not present.

Virulence. Completely avirulent for mice by mucin technique.

Stability. When fully dissociated, the rough form is very

stable over long periods of time. In intermediate stages of dissociation, the morphology is more variable. A strain which is in the process of changing from S → R may suddenly produce colonies which are much smoother than the colonies of the previous plating. With continued transfer in serum broth, however, the culture eventually goes over to the R phase. Concomitantly with this S → R shift, there is a more or less progressive loss of type-specificity, but in some instances a strain may retain slight traces of SSS after its colonial form has become distinctly rough. On the other hand, type-specificity may be lost at a stage when the strain is in an intermediate phase morphologically.

After prolonged cultivation on chocolate agar, many rough strains spontaneously give rise to a number of variants, in a few instances a smoother component than the original form, but most frequently one or more very rough or "degraded" forms. One such strain, a Type *b* when first isolated from a blood culture, produced (after artificial dissociation) a single type of rough colony which had all the characteristics of the rough phase. When plated out after two years' cultivation on artificial media, this strain produced three kinds of colonies. Since at least one of these variants has not, to the authors' knowledge, been described[2] before, a detailed description of the characteristics of all three variants is listed in table 1. For purposes of comparison, the characteristics of the original and the converted rough forms of the strain are also given in the table.

Reversibility of the R to the S or the M phase

The fact that smooth colonies may appear spontaneously in a culture which according to all criteria is in the rough phase has been stated previously. It has also been stated, however, that the spontaneous reversion of a smooth to a mucoid form has not been observed; nor has it been possible to bring about

[2] None of the photographs in Holster's (1931) excellent paper on variation in *H. influenzae* resembled this variant closely enough to permit its identification with any of the variants described.

TABLE 1

	ORIGINAL	CONVERTED ROUGH	VARIANT A	VARIANT B	VARIANT C (NEW)
Colonial morphology	Large, smooth, convex, opaque. (Fluorescence not recorded)	Medium size, very rough surface, edge irregular, bluish translucent non-fluorescent	Medium size, moderately rough surface, edge regular, bluish opaque, non-fluorescent	Very small, smooth, convex round, edge regular, translucent non-fluorescent	Tiny (just visible to the naked eye), very flat, very rough, very irregular edge, dull, transparent, non-fluorescent
Morphology of organisms	Short rods and coccobacilli; quite uniform in size and shape	Thick club forms, rods of all sizes, and filaments; marked pleomorphism	Fat coccobacilli, clubforms, few filaments; marked pleomorphism	Coccobacilli, club forms long rods; marked pleomorphism	All long, thin tangled filaments
Precipitin titre (vs type *b* antiserum)	1:8	0	1:2	0	0
Agglutinin titre (vs type *b* antiserum)	1:1280	0	1:640	1:640	1:640
Mouse virulence (in M.L.D.'s)	*	†	5,000,000		200,000,000

* The virulence of 11 similar strains determined at a later date varied from 30,000 to 1,600,000 M.L.D.'s.

† The virulence of 12 similar strains determined at a later date varied from 2 million to 7 billion M.L.D.'s.

this change by cultural manipulation. In an effort to determine whether or not the R → S → M transformation could be accomplished by animal inoculation, the following experiments were carried out:

a. Repeated mouse passage by the intraperitoneal route of a rough strain (in mucin) isolated from a case of meningitis secondary to trauma,

b. Repeated mouse passage by the intraperitoneal route of a rough strain (in mucin) derived from a mucoid, meningeal Type *b* strain,

c. Combined intraperitoneal and subcutaneous injection of a rough strain, originally isolated from the respiratory tract,

d. Combined intraperitoneal and subcutaneous injection of a mucoid, Type *b* filtrate, together with a rough strain, originally isolated from the respiratory tract,

e. Combined intraperitoneal and subcutaneous injection of a mucoid Type *b* vaccine, together with a rough strain, originally isolated from the respiratory tract.

In no instance was the transformation R → M accomplished by any of the above methods. On the other hand, the R → S conversion was brought about by every method but the last. In most instances, however, the S form did not maintain its stability, and after several transfers on artificial media reverted to the R form.

It is interesting to note that the R → S conversion took place very gradually in experiments (a) and (b), whereas in experiment (c) the change occurred abruptly. The progressive change in colonial form from R → S in (a) and (b) was accompanied by the appearance of "swollen" or "globoid" forms in smears of the peritoneal exudates. After twenty to thirty mouse passages, the "swollen" forms were replaced by coccobacilli, uniform in size and staining properties. In experiment (c), in which the R → S change occurred abruptly, the S colonies appeared on plates streaked with pus from the subcutaneous abscess. Smears of the same exudate showed no "swollen" forms, but only coccobacilli which were uniform in size and staining properties.

A summary of the characteristics of the three phases of dissociation in *H. influenzae* is given in table 2.

TABLE 2

Variation in Hemophilus influenzae

Morphology of colonies	Mucoid (fluorescent)	Smooth (non-fluorescent)	Rough (non-fluorescent)
Morphology of organisms	Coccobacilli	Coccobacilli or short rods; pleomorphism slight	Long rods, clubs, long tangled filaments; pleomorphism marked
Capsules	+	0	0
Growth in peptic-digest broth	Diffuse	Diffuse	Flocculent
Type specificity	+	0	0
Virulence:			
Human	+ or ±	±	0
Mouse	+ or 0	± or 0	0

DISCUSSION

Morphologically and culturally, the mucoid, smooth and rough phases of *H. influenzae* correspond very closely to the mucoid, smooth and rough phases of other organisms. Mucoid strains, which are highly virulent for man, produce a soluble polysaccharide and form a homogeneous group serologically (Type *b*). Mucoid strains less frequently associated with infection in man also produce a soluble polysaccharide, but in general fall into different serological types (a, c, d, e, f).

Smooth strains are characterized by their morphology, lack of type specificity and relative avirulence for man and animal. It should be emphasized that the "S" in the old classification is the M or mucoid as here classified. The S type of the present terminology is found in non-specific respiratory infections, sinusitis, bronchiectasis, etc., and has little if any pathogenic significance.

Rough strains are occasionally isolated directly from man, but are probably not associated with infection. Fully dissociated rough strains are, in general, only found in old, degraded stock cultures, or are derived from mucoid strains by experi-

mental methods. They are characterized by their morphology, lack of type specificity and avirulence for man and animal. They can be distinguished from smooth strains only by the appearance of their colonies and by the morphology of the individual bacteria.

It should be emphasized at this point that, whereas each variant phase can usually be distinguished by its serologic and pathogenic, as well as by its morphologic properties, the basis of differentiation ultimately depends on morphology. Dawson has pointed out that although all hemolytic streptococci which are virulent for mice are mucoid, capsulated and type-specific, not all mucoid strains are virulent for mice. In the authors' experience, this has also been true of *H. influenzae*. Strains which are highly virulent for mice are mucoid, fluorescent, capsulated and type-specific, but not all mucoid strains are mouse-virulent. Mucoid strains of Types *a* and *d*, for example, were found to be relatively avirulent for mice, although morphologically they were in the mucoid phase.

The M → S → R transformation may occur spontaneously in old stock cultures or can be brought about by ageing or by repeated transfer in homologous, type-specific, immune serume broth. The R → S conversion may also occur spontaneously, but probably happens rarely. R → S conversion can be induced by animal inoculation. This transformation may be brought about gradually by repeated mouse-passage intraperitoneally, or abruptly by means of combined subcutaneous and intraperitoneal inoculation. In the authors' experience, it has been impossible to effect the transformation R → M or S → M by animal inoculation, either by the intraperitoneal or subcutaneous routes.

Pittman succeeded in converting one R strain to the "S" (M) form by repeated growth in anti-R serum broth and by animal inoculation. The fact that this particular rough strain had been derived from a "smooth" (mucoid) strain only a short time before, suggests the possibility that the R variant might not have been completely converted to the R phase and therefore still retained some mucoid elements. This possibility is also suggested by the fact that Todd and Lancefield (1928) in their early studies on the dissociation of the hemolytic streptococcus

found that it was impossible to transform the glossy (smooth) into the matt-virulent (mucoid) form unless the glossy culture retained a small amount of type-specific substance.

Kun and Fenyvessy (1932) also had difficulty in effecting the R → S conversion in *H. influenzae*. They found, for example, that it was impossible to produce a stable S form from an R strain either by cultural manipulation or by intraperitoneal inoculation. The S strains so derived rapidly reverted to the R phase after a few transfers on chocolate agar. By subcutaneous injection, however, they were able to convert rough, "filament forming" strains into smooth "coccobacilli forming" strains which retained their smooth characteristics on artificial media. The increase in rat virulence and the changes in morphology exhibited by these derived strains indicate that transformation from the rough to the smooth phase took place. There is no evidence to indicate, however, that conversion to the mucoid phase was accomplished.

From the present observations and those of other investigators, it therefore appears that *H. influenzae* exhibits a three-phase dissociative pattern, namely, the mucoid, the smooth and the rough. The M → S → R transformation can be readily accomplished, whereas the R → S conversion can probably be obtained only by animal inoculation. The R → M transformation cannot be brought about by the techniques in use at this time.

SUMMARY

1. The mucoid, smooth and rough phases of *Hemophilus influenzae* are described.

2. The interconvertibility of the three dissociative phases is discussed.

3. The pattern of variation in *Hemophilus influenzae* corresponds to that of other micro-organisms.

REFERENCES

CHANDLER, C. A., FOTHERGILL, L. D., AND DINGLE, J. D. 1937 Studies on Haemophilus Influenzae. II. A comparative study of the virulence of smooth, rough, and respiratory strains of haemophilus influenzae as determined by infection of mice with mucin suspensions of the organism. J. Exp. Med., **66**, 789–799.

DAWSON, M. H. 1934 Variation in the pneumococcus. J. Path. and Bact., **39,** 323–344.

DAWSON, M. H., HOBBY, G. L., AND OLMSTEAD, M. 1938 Variation in the hemolytic streptococci. J. Inf. Dis., **62,** 138–168.

FOTHERGILL, L. D., AND CHANDLER, C. A. 1936 Observations on the dissociation of meningitic strains of H. influenzae. J. Immunol., **31,** 401–415.

FOTHERGILL, L. D., DINGLE, J. D., AND CHANDLER, C. A. 1937 Studies on Haemophilus Influenzae. I. Infection of mice with mucin suspensions of the organism. J. Exp. Med., **65,** 721–734.

FOTHERGILL, L. D., AND WRIGHT, JOYCE 1933 Influenzal meningitis. The relation of age incidence to the bactericidal power of blood against the causal organism. J. Immunol., **24,** 273–284.

HADLEY, P. 1937 Further advances in the study of microbic dissociation. J. Inf. Dis., **60,** 129–192.

HOLSTER, S. G. 1931 The microbic dissociation of hemophilus influenzae. Yale J. Biol. & Med., **3,** 321–340.

HUNTINGTON, R. W. JR. 1935 On the behavior of hemophilus influenzae in certain diseases of children. J. Clin. Invest., **14,** 459–464.

KUN, L., AND FENYVESSY, V. B. 1932 Variabilität des Pfeifferschen Influenzabazillus im Tierkörper und ihre epidemiologische Bedeutung. Centralbl. f. Bakt., **124,** 485–496.

LEVINTHAL, N., AND FERNBACH, H. 1922 Morphologische Studien an Influenzabacillen und das ätiologische Grippeproblem. Ztschr. f. Hyg., **96,** 456–519.

PITTMAN, MARGARET. 1931 Variation and type specificity in the bacterial species hemophilus influenzae. J. Exp. Med., **53,** 471–492.

PLATT, A. E. 1937 A serological study of haemophilus influenzae. J. Hyg., **37,** 98–107.

TODD, E. W., AND LANCEFIELD, R. C. 1928 Variants of hemolytic streptococci; their relation to type-specific substance, virulence, and toxin. J. Exp. Med., **48,** 751–790.

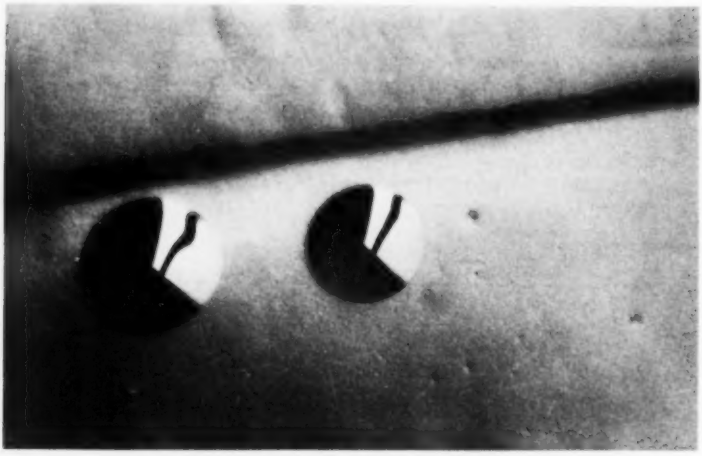


FIG. 1. MUCOID COLONIES
Magnification approximately 10 ×

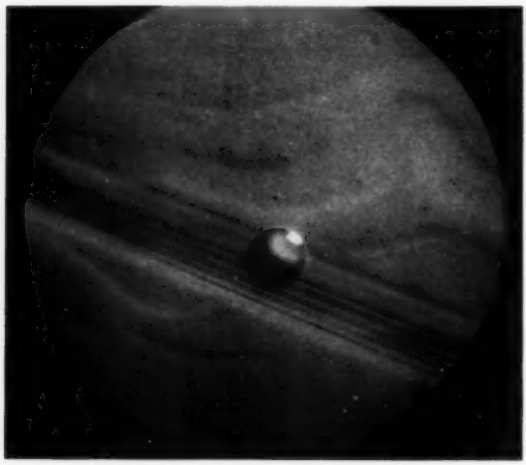


FIG. 2. A SMOOTH COLONY
Magnification approximately 10 ×

FIG. 3. ROUGH COLONIES
Magnification approximately 10 ×

THE NUTRITIONAL REQUIREMENTS OF CLOSTRIDIUM PARABOTULINUM, A

S. S. ELBERG AND K. F. MEYER

George Williams Hooper Foundation and Department of Bacteriology, Medical School, San Francisco, California

Received for publication October 13, 1938

This investigation was undertaken with the purpose of developing a synthetic medium for *Clostridium parabotulinum, type A*, in order to study certain phases of the toxin-producing mechanism.

The results mainly confirmed the findings of Fildes and his coworkers (1933) with respect to the essential nature of certain substances in yeast and pregnancy urines. In addition, certain evidence is presented which indicates that other substances besides the "sporogenes vitamin" are required for growth of *C. parabotulinum* in amino acid mixtures. The nature of these materials will be the subject of subsequent notes.

HISTORICAL INTRODUCTION

Studies on the simplification of media for the cultivation of *Clostridium botulinum* have not often been the primary purpose of researches on this organism. In fact, it is only in recent years that synthetic media have been attained for some members of the obligate anaerobes, notably *Clostridium sporogenes*.

That the complexity of the nutritional requirements of *C. botulinum* and the need for accessory growth factors were realized is stated in a paper by Wagner, Meyer and Dozier (1925). Hosoya and Kishino (1925) demonstrated the importance of the sulfhydryl group in the growth requirements of *C. botulinum* and found that cysteine adequately supplemented deficient tryptic digests of gelatin. Along the same lines, Quastel and Stephenson (1926) found that the lack of reduced sulfur com-

pounds was the limiting factor in the inadequacy of acid and tryptic digests of gelatin, although similar digests of casein did support growth.

Anderson (1928) then attempted unsuccessfully to simplify the routine media for *C. botulinum* by using nucleic acids, ammonium compounds, and mixtures of many amino acids as sources of nitrogen.

In a specific attempt to determine the essential amino acids for growth and toxin production by *C. botulinum*, Burrows (1932) found that acid hydrolysates of casein, if supplemented with tryptophane, would support growth of A and B strains. Such hydrolysates were rendered unfit, by treatment with Norite or by filtration through Seitz pads. Burrows (1933) then reported the successful substitution of an amino acid mixture for the protein hydrolysate and stated that cystine, proline, and leucine were essential acids for *C. botulinum*.

A very intensive study on the nutritional requirements of *C. sporogenes* was summarized in a report by Knight and Fildes (1933) in which was detailed the preparation and proof of the essential nature of a substance in yeast and pregnancy urines. Furthermore tryptophane was found to be an essential amino acid for growth of *C. sporogenes*. These authors also determined that the "sporogenes vitamin" and tryptophane were essential for the growth of *C. botulinum*.

Burrows (1934) reinvestigated the tryptophane requirements of *C. botulinum* and found that this organism differed from *C. sporogenes* both in the tryptophane and "sporogenes vitamin" requirements.

Fildes (1935) returned to the study of the requirements of *C. botulinum*, confirmed his former work and reaffirmed the belief that the difficulty lay in the use by Burrows of impure preparations of amino acids containing tryptophane as an impurity.

Fildes and Richardson (1935) working with *C. sporogenes* finally succeeded in replacing the gelatin hydrolysate by a known mixture of amino acids, most of which had been synthesized, and all of which had been recrystallized several times. They concluded that tryptophane, leucine, phenylalanine, tyrosine and

arginine were indispensable amino acids, while for adequate growth and maintenance of the cultures valine, cystine, methionine, and histidine were required.

Stickland (1934, 1935a, 1935b) cleared up the problem of the source of energy for the organism growing in a medium composed chiefly of amino acids by showing that *C. sporogenes* is able to activate certain pairs of amino acids, one being oxidized, the other reduced.

As to the nature of the "sporogenes vitamin" Pappenheimer (1935) found that it was an acid (pK^1 4.7), whose empirical formula was $C_{11}H_{14}O_4$, that it was most stable as the methyl ester, and that it contained one double bond and one hydroxyl group.

Following this there appeared a paper by Hosoya, Kuwashima, Kayo, Oda, and Kagabe (1936) on a factor essential for "pathogenic bacteria." The source of their active preparation was an "aqueous extract of scomber." In amounts of 5×10^{-6} gamma per 10 ml. gelatin hydrolysate basal medium, growth of *C. botulinum* occurred. The paper deals mainly with the preparative aspects, while information concerning the methods of assay or nature of the material is lacking.

METHODS

Media

(a) Isolation of colonies and purification of strains from stock cultures were effected on blood plates prepared as recommended by Schoenholz (1928).

For carrying stock cultures a beef-heart medium was employed in which Neopeptone was used as the principal nitrogenous ingredient. The inclusion of minced pieces of beef heart at the bottom of the tubes made the use of vaseline seals unnecessary.

For the production of spores a casein-digest medium recommended by Sommer (1930) was used.

A medium which was found to initiate excellent growth, toxin production and enzyme formation contained a filtered infusion of beef heart to which were added 1 per cent glucose, 0.5 per

cent monobasic potassium phosphate and 4 per cent Difco proteose peptone. The final pH was adjusted to 7.4. Sterile petrolatum served as an efficient seal.

Inoculum

Several liters of spores of a carefully purified strain of *C. parabotulinum, type A*, were washed five times in sterile M/15 phosphate buffer, pH 7.0 and eventually suspended in sterile double-distilled water. After the purity and viability of the spores were tested, the suspension was heated at 80°C. for forty-five minutes, cooled to room temperature and aseptically removed in 25 cc. lots to a Flosdorf-Mudd "Lyophile" apparatus (1935). The spores were rapidly desiccated from the frozen state under high vaccum and came out of the apparatus in the form of a white powder, perfectly viable and toxigenic. They were stored in small vials and checked for purity, viability and toxigenicity at monthly intervals.

Preparation of protein hydrolysates

Gelatin and casein, having been shown to support growth of *C. botulinum* under specified conditions, were selected to supply the principal nitrogenous substances in the media to be simplified.

(a) Nelson's photographic gelatin was prepared in the hydrolyzed form as recommended by Fildes (1935).

(b) Washed isoelectric gelatin (Peter Cooper) was prepared according to the method of Northrop and Kunitz (1928) and hydrolyzed by refluxing eight hours with five times its weight of sulfuric acid in 30 per cent solution. The acid was exactly removed with baryta, and the filtrate concentrated *in vacuo* to a thick syrup.

(c) Unwashed Peter Cooper gelatin was hydrolyzed in the same way as (b) and worked up to a syrup.

(d) Casein (Schering-Kahlbaum, according to Hammarsten) was refluxed thirty-six hours with 30 per cent sulfuric and worked up to a syrup as in the previous cases.

"Vitamin" preparation

In the main the original procedure used to prepare the "sporogenes vitamin" followed that of Knight and Fildes (1935a). The active preparation from brewer's yeast was such that 1×10^{-6} gram activated 10 ml. of a deficient medium. The final product was diluted so that 0.1 cc. contained 1×10^{-6} gram of dry material.

EXPERIMENTAL

The medium upon which it was desired to improve was that proposed by Fildes (1935) for *C. botulinum*. It was made up as follows:

Sodium citrate	3.0	grams
Monobasic potassium phosphate	4.5	grams
Water	500	cc.
1N Sodium Hydroxide	26	cc.
Dissolve and add:		
l-cystine	0.05	grams
l-tyrosine	0.05	grams
dl-valine	0.10	grams
Acid hydrolyzed protein (5 per cent solution)	50	cc.
Dissolve; dilute to 600 cc.; pH to 7.4; tube in 6 cc. amounts; autoclave at 121°C. for 20 minutes.		
Before the inoculum was added, all tubes received the following:		
Magnesium sulfate (0.4 per cent solution)	0.1	cc.
Tryptophane (0.2 per cent solution)	0.1	cc.
Sporogenes vitamin	0.1	cc.
1N Sodium Hydroxide	0.25	cc.
Thioglycollic acid (1 per cent in 1N hydrochloric acid)	0.20	cc.
Water to	9.0	cc.
Inoculum (0.01 mgm. dried spores in 100 cc.)	1.0	cc.

All of the above components were sterilized in the autoclave. The amino acids and salts were purified by recrystallization 3–5 times before use. The tubes were plugged with cotton wrapped in washed gauze. The thioglycollic acid was used as purchased. The acid-hydrolyzed protein was diluted so that it was 5 per cent based on the weight of the dry protein used. All incubations were carried out in a phosphorus jar at 37°C.

Experiment 1

The first few experiments on this type of medium were entirely confirmatory in nature, namely, to determine for the strain of *C. parabotulinum* selected the necessity for tryptophane and the "sporogenes vitamin" as previously reported (Burrows, 1932, 1933; Fildes, 1935).

The results indicated that *C. parabotulinum* did require these two components in the hydrolysate medium before any growth would occur. Toxin was produced in ninety-six hours following noticeable autolysis. The occurrence of lysis around the seventy-second hour of incubation was a regular sequel to growth in this type of medium and decreased the usefulness of the medium.

TABLE 1

Growth-supporting ability of protein hydrolysate (medium used as on page 5 but without added cystine, tyrosine and valine)

TUBE	INCUBATION PERIOD		TOXIN 96 HOURS
	24 hours	48 hours	
1 Basal medium + Nelson photographic gelatin hydrolysate	+++	++++	+
2 Basal medium + Peter Cooper gelatin hydrolysate	+++	++++	+
3 Basal medium + Isoelectric gelatin hydrolysate	+++	++++	+
4 Basal medium + Kahlbaum casein hydrolysate	+++	++++	+
5 Basal medium + Egg albumin hydrolysate	++	++++	+
6 Basal medium + Water to volume	0	0	0

Experiment 2

A comparison between the efficacy of acid hydrolysates of gelatin, casein and egg albumen indicated that there was little difference between the abilities of these proteins to support growth in spite of the differences in their amino acid contents.

The slight inferiority of egg albumin was rechecked and confirmed. Efforts to base this on the serine and oxyproline deficiencies of egg albumin by adding these amino acids in amounts equivalent to their respective concentrations in gelatin and casein did not improve the ability of egg albumin to support

growth. Since casein and gelatin supported growth in spite of the many differences in amino acid content (glycine, valine and tyrosine, particularly), it was decided to determine the nature of the substances present in the isoelectric gelatin which was suitable for the growth of the organisms.

Experiment 3. Fractionation of gelatin hydrolysate

The method which gave the best fractionation of the acid hydrolysate of isoelectric gelatin was one involving the formation of the copper salts of the amino acids and the subsequent separation of these salts based on their solubilities in methyl alcohol and water (Towne, 1928, 1936).

Three fractions were separated which were (I) the alcohol-soluble water-soluble group including proline, hydroxyproline, isoleucine, valine and the "hydroxyvaline" of Schryver, (II) the alcohol-insoluble water-soluble group including glycine, serine, alanine, arginine, histidine, lysine and pyrrolidone compounds, (III) the alcohol-insoluble water-insoluble group which includes leucine, phenylalanine, tyrosine and cystine.

The fractions were tested for their growth supporting ability in a deficient medium composed of tryptophane, sporogenes vitamin, thioglycollic acid, magnesium sulfate, and sodium hydroxide at pH 7.4. (The amount of each component is the same as that used in the other tests and is summarized above.)

The results indicate that the alcohol-insoluble water-soluble fraction of amino acids is sufficient to support growth of *C. botulinum*. Apparently, from a mixture containing glycine, serine, alanine, arginine, lysine, and histidine, supplemented with tryptophane and sporogenes vitamin, energy and structural components were minimally satisfied.

The addition of fraction I to II did not materially increase the amount of growth nor in fact did it stimulate an earlier germination of the spores. The increase in the amount and the earlier germination in the presence of fractions II + III and I + II + III was paralleled by the same increase when the amount of II in the medium was doubled or tripled.

Although there are several ways in which these results can be

explained, the most attractive approach is by way of the Stickland reaction (1934), in which the source of the energy available to the organisms growing in such a medium is based on reactions between pairs of amino acid molecules.

Fraction II contains alanine which has been shown to be activated as a hydrogen donator by *C. sporogenes*, (Stickland, 1934) plus glycine and serine, the reducible components in the Stickland reaction. This might be one system open to *C. botulinum*. Furthermore, when Fraction III was added to II an initially larger amount of growth appeared which did not

TABLE 2

Effect of gelatin hydrolysate fractions on growth of C. parabotulinum

TUBE	24 HOURS	42 HOURS	96 HOURS
1 Medium + 0.25 cc. Fraction I	0	0	0
2 Medium + 0.25 cc. Fraction II	++	+++	++
3 Medium + 0.25 cc. Fraction III	0	0	0
4 Medium + 0.50 cc. I	0	0	0
5 Medium + 0.50 cc. II	+++	++++	+++
6 Medium + 0.50 cc. III	0	0	0
7 Medium + 0.25 cc. I + 0.25 cc. II	++	+++	++
8 Medium + 0.25 cc. I + 0.25 cc. III	+	+	+
9 Medium + 0.25 cc. II + 0.25 cc. III	+++	++++	+++
10 Medium + 0.25 cc. I + 0.25 cc. II + 0.25 cc. III	+++	++++	+++
11 Medium + 0.75 cc. I	0	0	0
12 Medium + 0.75 cc. II	+++	++++	+++
13 Medium + 0.75 cc. III	0	0	0
14 Medium + 0.50 cc. water	0	0	0

materially increase over that finally obtained with II alone. Fraction III contained leucine and tryosine among other acids from the hydrolysate. Stickland (1934) and Knight and Fildes (1935) reported that, for *C. sporogenes*, leucine is a more readily available oxidizable component than alanine. Fraction III, supplying as it does acids which may be more available in the case of *C. sporogenes*, may act similarly in the case of *C. botulinum*. It remains to test the various amino acids which can be activated by *C. botulinum* to act as donators and acceptors of hydrogen, before carrying over to this organism explanations which may apply only to *C. sporogenes*.

It is also realized that the fractionation of protein hydrolysates by the copper salt method must involve a certain lapping over of fractional components because of faulty technique. If this occurs the magnitude of the concentrations of an amino acid for example may well be within the range of growth-supporting function as demonstrated by Fildes (1935). This would also explain the growth obtained with Fraction II alone.

The amino acids known to be in the various fractions of the protein hydrolysate were substituted for these fractions and tested with a new "sporogenes vitamin" preparation.

The new batch of "sporogenes vitamin" was prepared as previously but with the alteration from the method of Knight and Fildes (1933) that 0.4 per cent hydrochloric acid in methyl alcohol was used as the initial extracting solvent. The extractions were carried out for four days, using fresh changes of solvent, after which the alcohol fractions were evaporated *in vacuo* to dryness, the residue dissolved in hot water, boiled a few minutes and centrifuged. After the supernatant fluid was again concentrated *in vacuo* to a small volume, the steps followed those of Knight and Fildes.

About two hundred milligrams of residual material were obtained from the yeast of which the potency tests in gelatin hydrolysate basal medium revealed that 3.3×10^{-8} gram activated 1 ml. of the deficient medium (detailed on page 433).

Experiment 4

Several different lots of amino acid mixtures were prepared. Lot 1 was composed only of the amino acids known to be contained in the "Towne Fractions II and III." The amounts of each acid in this lot were based on the best available analyses of the amino acids in gelatin. This was further corrected for the amount of hydrolysate, or original protein hydrolysed, which had been shown to support growth.

Lot 2 was more inclusive, containing all the amino acids thought to be in gelatin, and again the amounts of each were based on the reported percentages in gelatin (Bodansky, 1931).

Lot 3 was the mixture developed by Fildes and Richardson (1935) for the growth of *C. sporogenes*.

A brief summary of the mixtures is presented in table 3.

Of those which were not synthetic, the naturally occurring form, purified by recrystallization was selected.

The amino acids were dissolved and added to the following basal mixture:

Monobasic potassium phosphate	4.5 grams
1N Sodium Hydroxide	25 cc.
Water	500 cc.

Adjusted to pH 7.4, diluted to 600 cc., tubed in 6 cc. amounts and autoclaved

Each tube received the necessary amounts of magnesium sulfate, thioglycollic acid, and the test amount of vitamin preparation previously adjusted to pH 7.4 with 1N sodium hydroxide. Total volume was brought to 9 cc. The inoculum, 1 cc. of a spore suspension, brought the volume to 10 cc.

These media were tested with both available preparations of sporogenes vitamin, the old one, now deteriorating in potency towards gelatin hydrolysate media, and the fresh preparation.

A summary of the comparative activities of the two preparations in the various media is tabulated in table 4.

(1) It will be seen that the old preparation of sporogenes vitamin was completely inactive towards the amino mixtures, whereas the fresh preparation supported a moderate amount of growth in this type of medium. It is also evident in the table above that the old preparation was less effective in the protein hydrolysate medium.

(2) The second point indicated is the loss of activity of the new "vitamin" preparation towards the amino acid mixtures, after ageing about a week.

This loss of potency was by no means an "all or none affair" but was a gradual loss, demonstrable by increasing amounts of preparation required for equivalent turbidities.

(3) The third lot of amino acids was far superior to the other two batches, indicating a striking similarity in growth requirements between *C. sporogenes* and *C. botulinum*.

(4) It was impossible to replace the "Towne Fractions II and

III" by the amino acids known to be contained therein, suggesting the presence of other substances or the overlapping of the fractions.

TABLE 3

AMINO ACID	PER CENT IN GELATIN (BODANSKY)	GRAMS PER LITER		
		Lot 1	Lot 2	Lot 3
Glycine	25.4	0.029	0.029	0.2
s-alanine	8.7	0.085	0.085	0.12
s-leucine	7.1	0.624	0.624	0.17
l-proline	9.5		0.836	0.15
l-oxyproline	14.1		1.24	
s-phenylalanine	1.4	0.123	0.123	0.08
s-glutamic	5.8		0.51	
s-aspartic	3.4		0.308	0.18
s-serine	0.4		0.035	0.14
l-tyrosine	0.01	0.0008	0.0008	0.05
l-histidine	0.9	0.0792	0.0792	0.05
d-arginine	8.2	0.76	0.76	0.05
s-lysine	5.9	0.51	0.51	0.09
l-tryptophane	0.0	0.02	0.02	0.02
l-cystine	0.2			0.06
s-valine				0.15
s-methionine				0.07

The acids marked "s-" were synthetic preparations obtained principally from Eastman Co., and Amino acid Mfgrs., U. C. L. A.

TABLE 4

Effect of sporogenes vitamin preparations

DAILY TEST	GELATIN HYDROLYSATE		AMINO ACID LOTS		
	Growth	Toxin	Number 1	Number 2	Number 3
Vitamin (prepared July, 1936)	++	+	0	0	0
Vitamin (prepared July, 1937)	++++++	+++	0	++	+++
After one week:					
Vitamin (July, 1936)	++	+	0	0	0
Vitamin (July, 1937)	++++++	+++	0	+	+

(5) Toxin was demonstrated in amino acid mixtures supporting growth, but was not particularly potent. One-half cubic centimeter of supernatant fluid from the cultures usually killed

unprotected mice within 36–48 hours, indicating a rather weak toxin. However, this comparison must be qualified because of the fact that growth in the amino acid mixtures, although very apparent, was nevertheless inferior.

One conceivable explanation of the results appeared to involve another essential substance present in gelatin and casein acid hydrolysates additive in effect to the yeast preparation. When the organisms were forced to use purified amino acids as their main food source, they required preformed a larger minimum amount of the "sporogenes vitamin," which could be supplied to them in workable volumes of fresh yeast preparations. Assuming that ageing and subsequent deterioration of the "vitamin" resulted in less than the critical amount being available, the organisms would then find it impossible to grow in such synthetic media. In the gelatin hydrolysate, however, enough "accessory factor" might be present to replace the material lost by ageing of the yeast preparation. A statement by Fildes (1935) that gelatin contains minute amounts of "sporogenes vitamin" seemed to support this hypothesis.

If some accessory factor is present in gelatin hydrolysates, sufficient in the presence of yeast preparation to supplement a deficient medium, the addition of small amounts of gelatin hydrolysate plus the yeast factor to amino acid mixtures should raise the concentration of essential material above the critical level and growth should occur.

When amounts of gelatin hydrolysate, sufficient in themselves to support merely a swirl of growth, were added to the amino acid mixtures, containing yeast preparation formerly sufficient to support growth, no additional growth resulted. When the concentration of hydrolysate was stepped up growth was visible, but the control tubes showed that this growth was due entirely to the hydrolysate and was independent of the amino acid base.

The following fractionations of the gelatin hydrolysate were attempted in order to produce a fraction supplementary only in activity and insufficient in itself to support growth.

1. Alcohol extraction

Ninety-five per cent alcohol was added to a portion of the hydrolysate until the precipitate no longer disappeared on heating. A minimum amount of water was added to dissolve the precipitate and the mixture placed in the ice box. After it had settled the precipitate was filtered and washed with alcohol. It was then dried and taken up in distilled water. The original filtrate containing the alcohol-soluble fraction was concentrated *in vacuo* and taken up in water. Growth tests in a deficient medium (page 433) indicated that the materials for growth were concentrated in the alcohol-soluble fraction since this fraction supported growth. The alcohol-insoluble fraction which did not support growth was also non-supplementary to the deteriorated sporogenes vitamin in the amino acid mixture number three.

2. Alcohol-ether extraction

The procedure adopted here had been used by Tatum and co-workers (1936) in their work on the potato fractions active for *Lactobacillus delbruckii*. Five volumes of 95 per cent alcohol were added to one volume of hydrolysate, then six volumes of ether were added. The mixture was shaken continuously while the ether was added. The precipitate was centrifuged down and taken up in distilled water. The alcohol-ether solution was concentrated, freed of organic solvents, and diluted to volume with distilled water. Only the alcohol-ether precipitate fraction, containing the dark material in the hydrolysate, contained essential growth materials, inasmuch as it supported growth in the absence of added amino acids. The alcohol-ether soluble fraction was negative alone and in the presence of added amino acids (mixture 3).

3. Treatment with lead acetate and ammonia

Although admittedly a remote possibility, it was decided to check the hydrolysates of gelatin and casein for the presence of accessory substances hydrolyzable to glucose. The hydrolysates

were first alkalinized with excess ammonia. A saturated solution of lead acetate was added until no further precipitation occurred. The filtrate was boiled to remove ammonia, saturated with hydrogen sulfide to remove the lead, boiled to remove excess hydrogen sulfide and brought to volume. The lead acetate precipitable fraction was taken up in a little water, saturated with hydrogen sulfide to remove the lead as before and worked up to volume. The growth-essential materials of the hydrolysate were contained in the lead-acetate-soluble fraction which alone supported growth. The insoluble fraction was of no value.

4. Treatment with norite

A portion of the original hydrolysate and also an aliquot of the lead-acetate-soluble fraction were treated with purified animal charcoal, boiled for an hour and filtered twice through Whatman #40 paper. The charcoal precipitates were washed twice with hot water and then dried. The filtrates and washings were combined and concentrated on the water bath to their original volume. Growth tests revealed that the essential materials had been completely removed by the norite.

Boiling the charcoal for a few hours with 95 per cent ethyl alcohol eluted the growth materials with much loss involved. A fraction supplementary only in effect could not be obtained.

5. Effect of the Neuberg-Kerb reagent (1911)

The hydrolysate was treated with sufficient 10 per cent sodium carbonate to alkalinize it. To this, a 25 per cent solution of mercuric acetate was added until a permanent brick-red precipitate appeared. Five volumes of 95 per cent alcohol were added. The mixture was then filtered. The filtrate and precipitate were freed of mercury by hydrogen sulfide and concentrated to volume on the water bath. The growth-essential materials were concentrated in the mercury-insoluble fraction. The soluble fraction was negative in the growth tests.

All growth tests with the above fractions were carried out in two basal media, (1) one containing only monobasic potassium phosphate, sodium hydroxide, tryptophane, sporogenes vitamin

and thioglycollic acid in amounts as on page 433, (2) the other being amino acid mixture lot 3. The fractions under test were added in amounts equivalent to that of the original protein hydrolysate which supported growth.

The results are summarized in table 5.

The inability to produce a fraction which was only supplementary in action to the sporogenes vitamin (i.e., which acts only in basal medium number 2) is indicated by the above table.

It was therefore decided to prepare another lot of sporogenes

TABLE 5

Activity of gelatin hydrolysate fractions

FRACTION	INCUBATION PERIOD		TOXIN
	Basal medium 1	Basal medium 2	
Original hydrolysate	++++	++++	+
Alcohol-soluble fraction	+++	+++	+
Alcohol-insoluble fraction	0	0	0
Alcohol-ether soluble fraction	0	0	0
Alcohol-ether insoluble fraction	+++	+++	+
Lead acetate-ammonia filtrate	+++	+++	+
Lead acetate-ammonia precipitate	0	0	0
Norite filtrate, pH 7.0	0	0	0
Neuberg-Kerb filtrate	0	0	0
Neuberg-Kerb precipitate (0.5 cc.)	+	+	0
Neuberg-Kerb precipitate (1.0 cc.)	++	++	0
Neuberg-Kerb precipitate (3.0 cc.)	+++	+++	+
Ethyl alcohol eluate of Norite-adsorbed material	+++	+++	+

vitamin, this time using pregnancy urine as a source of the material and adopting Pappenheimer's published method (1935) for the latter stages of the work. The preliminary treatment of the urine was carried out in accordance with some suggestions kindly sent to me by Dr. Pappenheimer.

One hundred liters of freshly obtained pregnant cow's urine were concentrated to one-tenth of the original volume by distillation *in vacuo*. Equal volumes of normal sulfuric acid and butyl alcohol were added to the concentrate and boiled for twenty-four hours under a reflux condenser. The butyl alcohol layer was

removed and concentrated *in vacuo* to a thick tar. This was partially dissolved in pyridine. An equal volume of peroxide-free ether was added and the insoluble matter removed. The material was then extracted with concentrated acid and the acidified extract alkalinized with sodium carbonate. The rest of the procedure follows Pappenheimer's description (1935).

The yellow material obtained was very active, 1×10^{-7} gram activating 1 ml. of the amino acid basal mixture developed by Fildes for *C. sporogenes*. However, the growth obtained with this amount of vitamin, and with increasing amounts also, did not compare favorably with the growth which the same amount of vitamin supported in gelatin hydrolysate media. One or more essential substances appear to be required for the growth of *C. parabotulinum* in synthetic amino acid mixtures, in addition to the active substance in yeast and pregnancy urines. A further investigation of these findings is being carried on.

SUMMARY

1. Previous reports concerning the essential nature of certain acidic fractions from yeast and pregnancy urines in the growth of *Clostridium parabotulinum* have been confirmed.

2. Growth of *Clostridium parabotulinum* in media composed of amino acids has been obtained.

3. Although the nutritional requirements of *Clostridium parabotulinum* resemble those of *Clostridium sporogenes* in the group of amino acids required for growth, there are qualitative differences in their needs which have not yet been solved to the point of obtaining massive growth in synthetic media.

REFERENCES

ANDERSON, B. G.: Investigations on Clostridium botulinum (unpublished). 1928.

BODANSKY, MEYER: Introduction to Physiological Chemistry (2d edition). 1931, John Wiley and Sons, New York.

BURROWS, WILLIAM: Growth of Cl. botulinum on casein hydrolysate media Jour. Infect. Dis., 1932, **51**, 298-308.

BURROWS, WILLIAM: Growth of Cl. botulinum on synthetic media. Jour. Infect. Dis., 1933, **52**, 126-137.

BURROWS, WILLIAM: Growth stimulating properties of cystine and tryptophane. Jour. Infect. Dis., 1934, **54**, 164–170.

FILDES, PAUL: Tryptophane and sporogenes vitamin requirements of B. botulinus. Brit. Jour. Exper. Path., 1935, **16**, 309–314.

FILDES, PAUL, AND RICHARDSON, G. M.: Amino acids necessary for growth of Cl. sporogenes. Brit. Jour. Exper. Path., 1935, **16**, 326–335.

FLOSDORF, E., AND MUDD, S.: Procedure and apparatus for preservation in lyophile form of serum and other biological products. Jour. of Immunol., 1935, **29**, 389–425.

HOSOYA, S., AND KISHINO, S.: Sulfhydryl compounds in anaerobic metabolism. Scient. Rep. Govt. Inst. Infect. Dis., 1925, **4**, 123–127.

HOSOYA, S., KUWASHIMA, Y., KAYO, S., ODA, M., AND KAGABE, K.: On the isolation of the growth factor of pathogenic bacteria. Proc. Imp. Acad., 1936, **12**, 671–675.

KNIGHT, B. C. J. G., AND FILDES, P.: A vitamin necessary for Cl. sporogenes. Brit. Jour. Exper. Path., 1933a, **14**, 112–124.

KNIGHT, B. C. J. G., AND FILDES, P.: Tryptophane and the growth of bacteria. Brit. Jour. Exper. Path., 1933b, **14**, 343–348.

NORTHROP, J. H., AND KUNITZ, M.: Preparation of isoelectric gelatin. Jour. Gen. Physiol., 1938, **11**, 477–479.

QUASTEL, J. H., AND STEPHENSON, M.: Relation of Cl. sporogenes to oxygen. Biochem. Jour., 1926, **20**, 1125–1137.

PAPPENHEIMER, A. M., JR.: The nature of the sporogenes vitamin. Biochem. Jour., 1935, **29**, 2057–2063.

SCHOENHOLZ, P.: Surface colonies of Cl. botulinum on blood agar. Jour. Infect. Dis., 1928, **42**, 40–47.

SOMMER, E. WAGNER: Heat resistance of botulinus spores. Jour. Infect. Dis., 1930, **46**, 85–114.

STICKLAND, L. H.: Studies in metabolism of the strict anaerobes. Biochem. Jour., 1934, **28**, 1746–1759.

STICKLAND, L. H.: Reduction of proline by Cl. sporogenes. Biochem. Jour., 1935a, **29**, 288–290.

STICKLAND, L. H.: The oxidation of alanine by Cl. sporogenes. Biochem. Jour., 1935b, **29**, 889–896.

STICKLAND, L. H.: The reduction of glycine by Cl. sporogenes. Biochem. Jour., 1935c, **29**, 896–898.

TATUM, E., PETERSON, W., AND FRED, E.: Essential growth factors of the propionic acid bacteria. Jour. of Bact., 1936a, **32**, 157–166.

TATUM, E., WOOD, H., AND PETERSON, W.: Nature of the Neuberg precipitate fraction of potato. Jour. of Bact., 1936b, **32**, 167–174.

TOWNE, B. W.: Separation of amino acids by means of their copper salts. Biochem. Jour., 1928, **22**, 1083–1086.

TOWNE, B. W.: The separation of the amino acids by means of their copper salts. Biochem. Jour., 1936, **30**, 1837–1844.

WAGNER, E., MEYER, K. F., AND DOZIER, C. C.: Metabolism of B. botulinus in various media. Jour. of Bact., 1935, **10**, 321–412.

THE LETHAL ACTION OF SHORT ULTRAVIOLET RAYS ON SEVERAL COMMON PATHOGENIC BACTERIA

D. G. SHARP

Departments of Surgery and Bacteriology, Duke University School of Medicine and Duke Hospital

Received for publication October 7, 1938

INTRODUCTION

The inhibitory and lethal effects of certain of the shorter ultraviolet rays on bacteria and fungi have been known for about fifty years. Recently, interest has been revived in problems concerning bacteria floating in the air especially in relation to the sterilization of air as a protection against air-borne infections. (Wells and Wells, 1936 and 1938, Hart, 1937 and 1938, and Sharp, 1938). Since a quantitative evaluation of the resistance to radiation of most of the bacteria against which ultraviolet-ray protection is needed has not been made, such comparative study seems desirable.

Careful quantitative estimates of the energy lethal for various species of bacteria have been made by Coblentz and Fulton (1924) on *Escherichia coli*, Gates (1929 and 1930) on *Staphylococcus aureus* and *Escherichia coli*, Wyckoff (1931) on *Escherichia coli*, Ehrismann (1931) on *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens*, etc., and Hollaender and Claus (1936) on *Escherichia coli*. In the last paper, the results of the previous work are summarized. Through a comparison of the data obtained by different experimental methods, attempts are made to reduce all the results to the common denominator of lethal energy per bacterium. It is notable that, while all the studies cited include some of the same species of bacteria, the absolute lethal energies given for a particular wave length differ substantially.

Studies of spore-forming bacteria (*Bacillus subtilis* by Duggar and Hollaender, 1934 and *Bacillus megatherium* by Hercik, 1936) indicate that approximately twice as much energy is necessary to kill the spores as is needed for the vegetative forms.

In none of the studies in which absolute energy values have been measured has more than slight attention been given to the common pathogenic bacteria. The work of Dreyer and Campbell-Renton (1936) gives lethal curves for several pathogens but the light intensities were measured in arbitrary units and are therefore not directly comparable with other quantitative work.

The experiments reported here were concerned with the determination of the relative resistance of several pathogens, notably the more important pathogenic air contaminants, as well as *Staphylococcus albus*, *Escherichia coli*, and *Serratia marcescens*, to the unfiltered rays of a low-pressure mercury glow lamp[1] of the type used for sterilization of air in operating rooms. Its characteristics have been described elsewhere (Sharp, 1938) and will be reviewed only briefly here.

METHOD

The highly pathogenic nature of several of the species of bacteria studied makes measurement in air suspension dangerous. Previous work (Sharp, 1938) has shown that in the instance of *S. albus* the amount of energy necessary to kill 100 per cent in air suspension (presumably saturated air) is of the same order of magnitude as that given by the plate method. It was considered advisable to employ the latter method.

Standard nine-centimeter Petri plates containing solid media suitable for the growth of the organism under observation were seeded uniformly with bacteria in the following manner: A twenty-four-hour broth culture was diluted with normal saline solution until it contained about fifteen thousand organisms per cubic centimeter. Five cubic centimeters of this were pipetted on to the agar surface of each plate and kept in motion for twenty

[1] This lamp is manufactured by Westinghouse Electric and Manufacturing Co. under the name of "Sterilamp."

seconds; the plate was then inverted for about forty seconds to drain off the excess liquid. By this means 600 to 1,000 evenly distributed colonies could be obtained on each plate (fig. 1).

Prepared plates were exposed to ultraviolet rays in a frame (fig. 2) at a distance of 35 inches from a pair of radiation tubes and

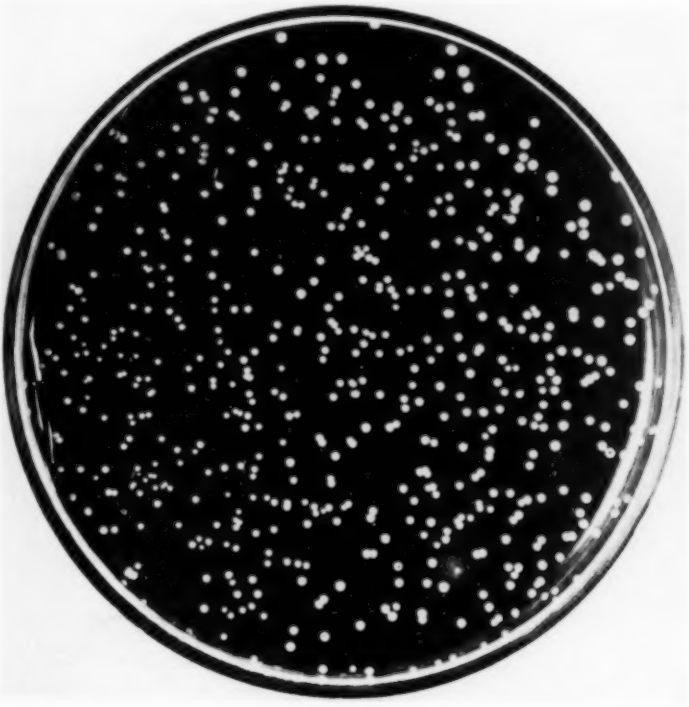


FIG. 1

in a plane normal to the rays. The tubes were 30 inches long, operated at about five degrees above room temperature and consumed 5 to 10 watts power each. They are particularly well adapted to this work since over 85 per cent of the total radiant output is in the resonance 2537 Å line. There are no other lines in the region below 3000 Å strong enough to have appreciable

bactericidal effect so that it is safe to assume, for present purposes, that the unfiltered rays are monochromatic and as such, comparable in their effect with those usually isolated with a monochrometer. This allows a direct comparison of results obtained here with those of other observers.

Immediately below the plates (fig. 2) and in a similar position with respect to the lamps was mounted a special ultraviolet dosimeter (Rentschler, 1930) consisting of a tantalum-target photoelectric cell and relay apparatus suitable for metering the energy received. This device recorded the actual amount of

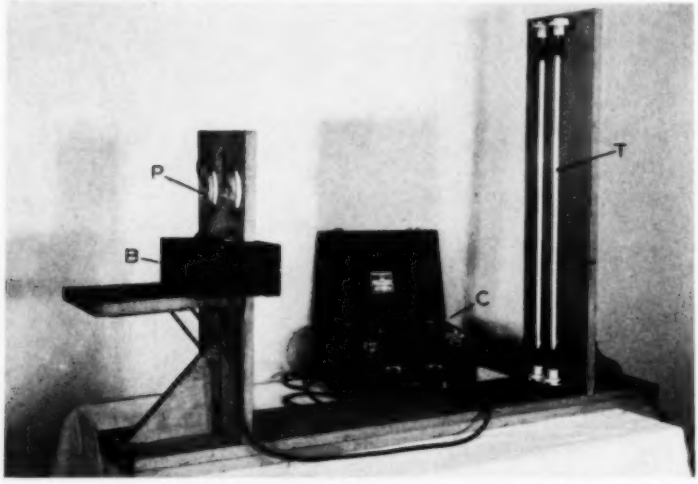


FIG. 2

energy received over a given length of time and so was free from errors that might have been caused by variation in lamp intensity. Standardization was effected by comparison with a bismuth-silver vacuum thermopile and National Bureau of Standards radiation constant. All energy values are given directly in incident ergs per square millimeter of exposed surface.

RESULTS

For each organism used, two separate groups of 12 to 15 plates were exposed on different days. Exposures were selected to give

the best possible determination of the amount of energy necessary to reduce the number of surviving bacteria to 10 per cent of the original number. No attempt has been made to examine closely the extremes of the reaction, namely; near 0 per cent and near 100

TABLE 1

ORGANISM	MEDIUM (pH 7.4)	INCIDENT ENERGY ERGS/MM² FOR 90 PER CENT KILLING (10 PER CENT SURVIVAL)	OTHER OBSERVATIONS
Bacillus anthracis (mixed spores and veg. forms)	Beef-extract agar	452	
Corynebacterium diphtheriae	Beef-infusion blood agar	337	
Staphylococcus aureus-hemolyticus	Beef-extract agar	260	218 ergs, Gates (1929) 600 ergs, *Ehrismann and Noethling (1931)
Escherichia coli	Beef-extract agar	245	150 ergs, *Ehrismann and Noethling (1931) 640 ergs, Wyckoff (1931)
Serratia marcescens	Beef-extract agar	220	82 ergs, *Ehrismann and Noethling (1931)
Streptococcus hemolyticus	Beef-extract blood agar	216	
Eberthella typhosa	Beef-extract agar	214	
Streptococcus viridans	Beef-extract blood agar	200	
Staphylococcus albus	Beef-extract agar	184	
Shigella paradysenteriae	Beef-extract agar	168	

Table showing incident energy at 2537 Å necessary to reduce several species of bacteria to a survival ratio of 10 per cent (90 per cent killed).

*Values based on 90 to 100 per cent killing.

per cent survival. This has been done by others, (Hollaender and Claus, 1936) for non-pathogenic bacteria, and it was not considered advisable to repeat it here since the exposure of many more plates would have been necessary.

Figures 3 to 12 show the logarithm of the survival ratio plotted against the incident energy in ergs per square millimeter and the

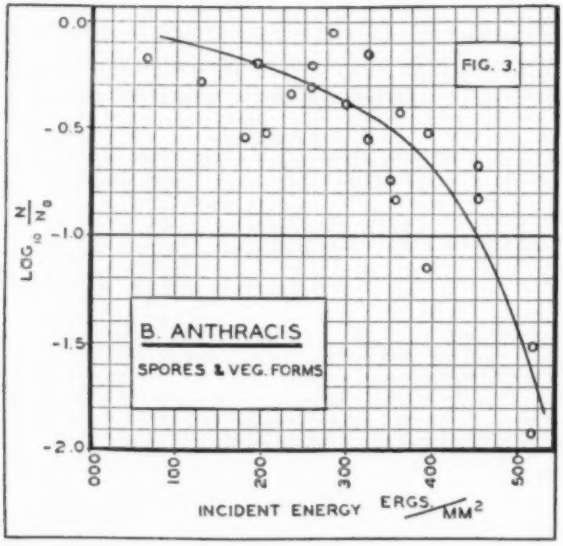


FIG. 3.
B. ANTHRACIS
SPORES & VEG. FORMS
$\log_{10} \frac{N}{N_0}$
0.0
-0.5
-1.0
-1.5
-2.0
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²

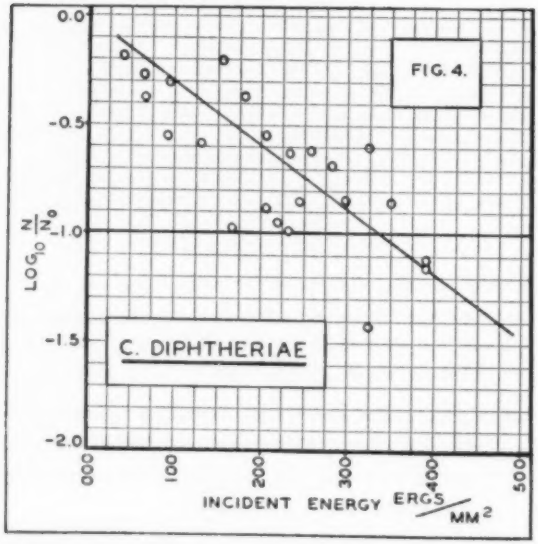
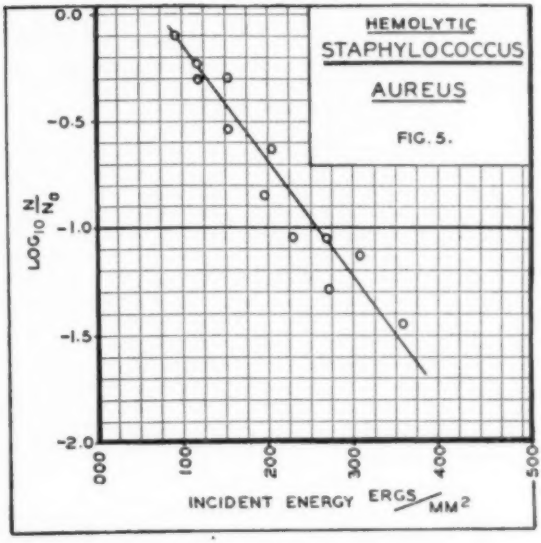
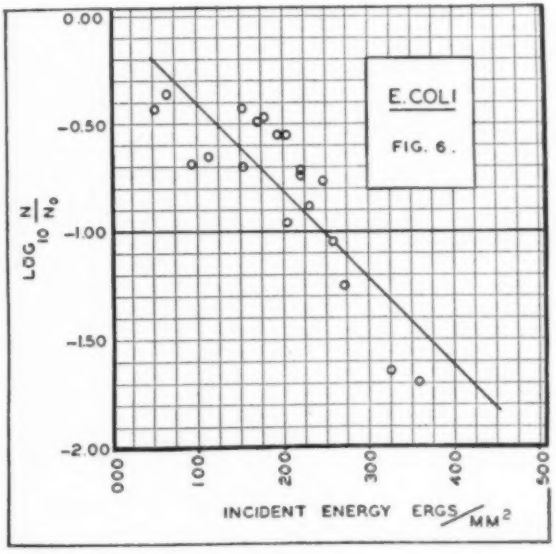
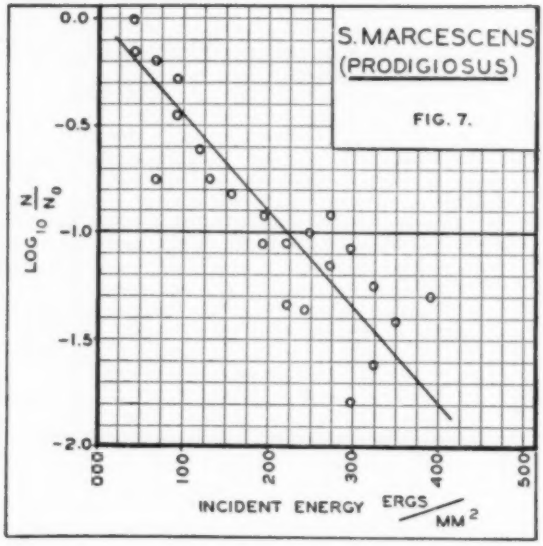
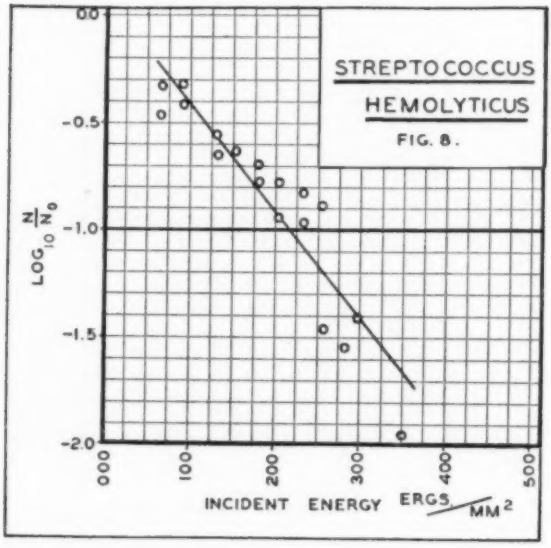


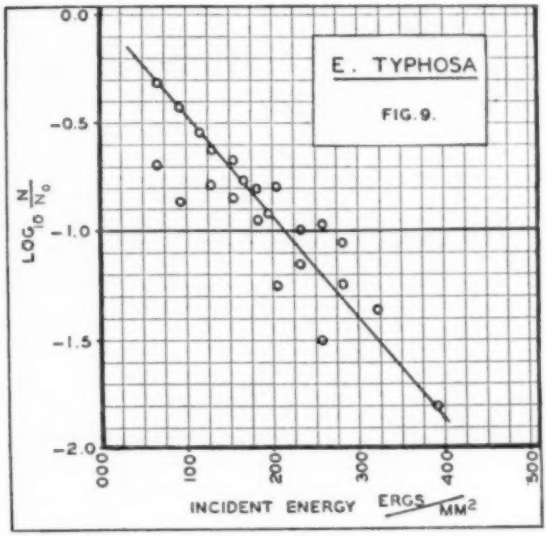
FIG. 4.
C. DIPHTHERIAE
$\log_{10} \frac{N}{N_0}$
0.0
-0.5
-1.0
-1.5
-2.0
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²

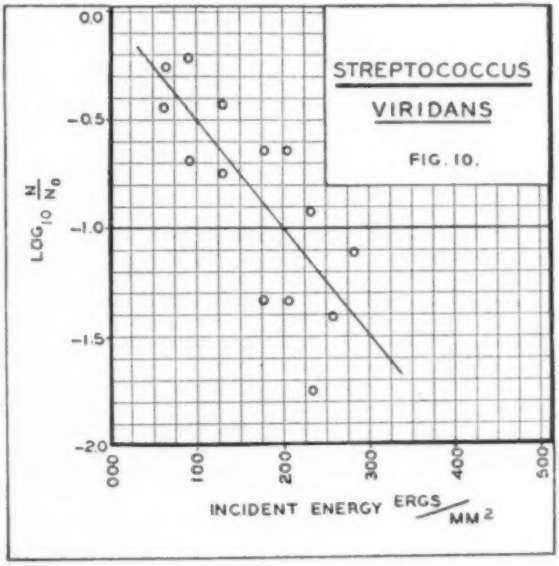

HEMOLYTIC
STAPHYLOCOCCUS
AUREUS
FIG. 5.
0.0
-0.5
-1.0
-1.5
-2.0
LOG10 N/N0
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM2

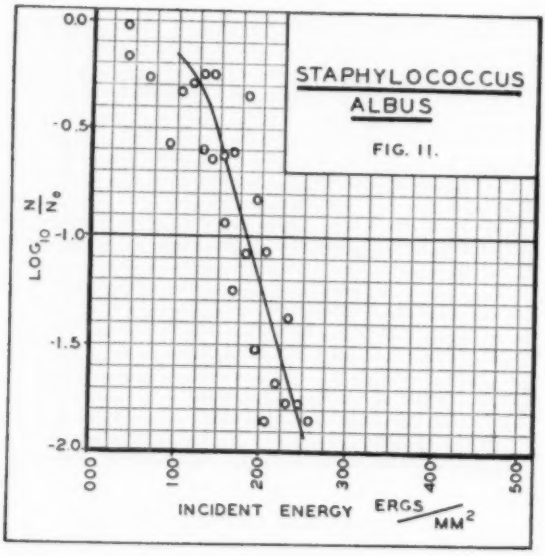

E. COLI
FIG. 6.
0.00
-0.50
-1.00
-1.50
-2.00
LOG10 N/N0
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM2

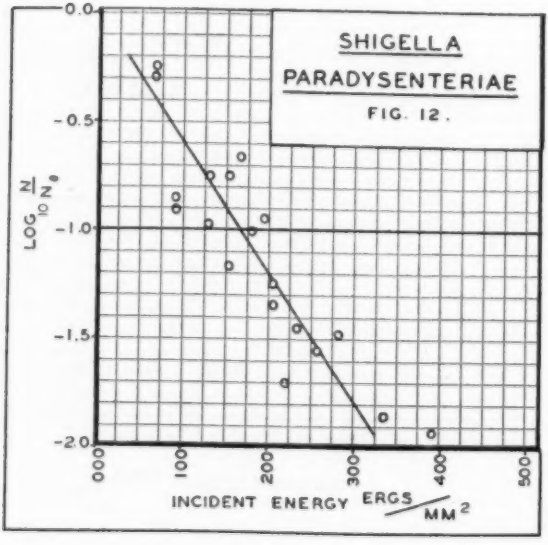

S. MARCESCENS
(PRODIGIOSUS)
FIG. 7.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} N/N_0$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²


STREPTOCOCCUS
HEMOLYTICUS
FIG. 8.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} N/N_0$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²


E. TYPHOSA
FIG. 9.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} \frac{N}{N_0}$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²


STREPTOCOCCUS
VIRIDANS
FIG. 10.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} \frac{N}{N_0}$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²


STAPHYLOCOCCUS
ALBUS
FIG. 11.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} N/N_0$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²


SHIGELLA
PARADYSENTERIAE
FIG. 12.
0.0
-0.5
-1.0
-1.5
-2.0
$\log_{10} N/N_0$
000
100
200
300
400
500
INCIDENT ENERGY ERGS/MM²

curves drawn through the points. The intersections of these curves with the ordinate line -1 (10 per cent survival) is the basis on which the bacteria are compared (table 1). The intensity was constant and such that the time required to give this amount of energy was about 2 minutes.

DISCUSSION

It has been shown for several types of bacteria, (Wyckoff, 1931, Hollaender and Claus, 1936, and Hercik, 1936), that the number dying per unit of incident energy is approximately in direct proportion to the number remaining alive in the culture, i.e.:

$$\frac{dN}{dE} = -k_1 N$$

E = energy incident on culture,
N = number of bacteria remaining after a given amount of energy has been applied.

$$\log \frac{N}{N_0} = -k_2 E$$

This, when integrated from energy $E = O$ to any given amount E, gives the corresponding survival ratio $\frac{N}{No}$.

No = number of bacteria originally on the plate as given by the controls.

The constant K_2 depends on the absorption coefficient of the particular species and on the wavelength of the light. Although certain deviations from this straight-line relationship between Log $\frac{N}{No}$ and the energy have been reported, it serves as a convenient method of comparison of species resistance and is used here to facilitate comparison with other data.

A direct comparison can be made in the case of *Staphylococcus aureus* with the data of Gates (1929) which show 218 ergs/mm^2 necessary to kill 90 per cent of these organisms on agar surface. From our data (fig. 5) it is seen that 260 ergs/mm^2 were necessary

for our strain of *S. aureus*. The same author (Gates, 1930) found 110 ergs/mm^2 necessary to reach 50 per cent killing of *E. coli* and our data (fig. 6) would indicate 75 ergs/mm^2 for this organism. Wyckoff (1931), on the other hand, found 640 ergs/mm^2 necessary for 90 per cent killing while curve 6 shows only 245 ergs/mm^2. Only approximate comparisons with the studies of Ehrismann and Noethling (1931) are possible since their estimates were made on the broad basis of 90 to 100 per cent killing. Their figures have been entered in table 1, and it will be seen that large variations occur. No such wide differences in resistance have been found among the various species tested here. In fact, all the figures for 90 per cent killing of non-sporing organisms fall between 168 ergs/mm^2 (*Shigella paradysenteriae*) and 337 ergs/mm^2 (*Corynebacterium diphtheriae*).

Bacillus anthracis, which showed more resistance than any of the nonsporing species, was killed by about twice the energy necessary to kill *E. coli*. This was true even if complete sterilization instead of 90 per cent killing was considered as the point of comparison. The results of Hercik (1937) on *Bacillus megatherium* indicate, too, about twice as much energy required to kill spores as was required for vegetative forms. The curve (fig. 3) seems to indicate by its increasing rate of fall that there is an apparent decrease in resistance rather than the expected increase due to surviving spores.

The results summarized in table 1 indicate that the resistance to 2537 Å rays of the several species of bacteria was not only of the same order of magnitude but that the most resistant non-spore producer (*C. diphtheriae*) was only about twice as resistant as the least resistant (*S. paradysenteriae*).

It is to be expected that the ultraviolet ray resistance of these bacteria in air suspension would be subject to certain variables not present in this experiment. Wells (1936) has reported wide differences in bacterial resistance with changes in the relative humidity of the air. His findings indicate a reduced resistance in air of lower humidity. In the light of his observation our previous work on *S. albus* done in air (Sharp 1938) under conditions near saturation could be taken to indicate the maximum

resistance of the organism. The present data taken from bacteria on moist plate surfaces might be subject to the same considerations. The lethal energies in both experiments were found to be of the same order of magnitude.

SUMMARY

The following species of bacteria were tested for resistance to short ultraviolet rays, and the energy necessary to reduce each to a 10 per cent survival ratio is recorded: *Eberthella typhosa*, *Shigella paradysenteriae*, *Corynebacterium diphtheriae*, *Staphylococcus aureus* (*Hem*), *Staphylococcus albus*, *Streptococcus viridans*, *Streptococcus hemolyticus*, *Bacillus anthracis* (mixture of spores and vegetative forms), *Escherichia coli* and *Serratia marcescens*.

The mixture of the spore and vegetative forms of *Bacillus anthracis* was not more than twice as resistant as *Escherichia coli*. This was true even when 100 per cent killing or zero survival was considered as a reference point.

Unfiltered radiation (85 per cent 2537 Å) from a commercial low pressure glass mercury arc was used. Its degree of purity was sufficiently great to make a monochrometer unnecessary for bacterial work, and regular 9 cm. Petri plates can be conveniently exposed to a uniform field of rays.

A commercial ultraviolet ray dosimeter, calibrated in absolute units, was used to measure the energy. The sensitive agent is a tantalum-target vacuum photoelectric cell.

It was found that the extreme values of energy necessary to kill 90 per cent of non-sporing organisms were 168 ergs/mm^2 (*Shigella paradysenteriae*) and 337 ergs/mm^2 (*Corynebacterium diphtheriae*). For a mixture of spores and vegetative forms of *Bacillus anthracis*, however, 452 ergs/mm^2 were necessary. The results obtained are compared with the data of other observers.

REFERENCES

Coblentz, W. W., and Fulton, H. R. 1924 A radiometric investigation of the germicidal action of ultra-violet radiation. Scientific paper of U. S. Bureau of Standards, no. 495, vol. 19.

Dreyer, Georges, and Campbell-Renton, M. L. 1936 A quantitative study of the action of ultra-violet light on bacteria. Proc. Roy. Soc. Ser. b, **120**, 447–472.

DUGGAR, B. M., AND HOLLAENDER, A. 1934 Irradiation of plant viruses and of micro-organisms with monochromatic light. Jour. Bact., **27**, 241–255.

EHRISMANN, O., AND NOETHLING, W. 1931 Über die baktericide Wirkung monochromatischen Lichtes. Zeit. f. Hygiene & Infk., **113**, 597–628.

GATES, F. L. 1929 A study of the bactericidal action of ultra violet light. Jour. Gen. Physiol., **13**, 231–260.

GATES, F. L. 1930 A study of the bactericidal action of ultra violet light. Jour. Gen. Physiol., **14**, 31–42.

HART, DERYL 1937 Sterilization of the air in the operating room by bactericidal radiant energy. Surgery, **1**, 770–784.

HART, DERYL, AND GARDNER, C. E. 1937 Sterilization of the air in the operative region with bactericidal radiant energy. Trans. Southern Surg. Assoc., **49**, 376–402.

HART, DERYL 1938 Sterilization of the air in the operating room with bactericidal radiation. Jour. Thoracic Surg., **7**, 525–535.

HERCIK, FERDINAND 1937 Action of ultraviolet light on spores and vegetative forms of *B. megatherium sp.* Jour. Gen. Physiol., **20**, 589–594.

HOLLAENDER, A., AND CLAUS, W. D. 1936 The bactericidal effect of ultraviolet radiation on *Escherichia coli* in liquid suspensions. Jour. Gen. Physiol., **19**, 753–765.

RENTSCHLER, H. C. 1930 Jour. Am. Int. Elec. Eng., **49**, 113.

SHARP, D. G. 1938 A quantitative method of determining the lethal effect of ultraviolet light on bacteria suspended in air. Jour. Bact., **35**, 589–599.

WELLS, W. F., AND WELLS, M. W. 1936 Air-borne infection. Jour. Amer. Med. Assoc., **107**, 1698–1703.

WELLS, W. F., AND WELLS, M. W. 1938 Measurement of sanitary ventilation. A. J. Pub. Health, **28**, 343–350.

WYCKOFF, R. W. G. 1931 The killing of colon bacilli by ultra-violet light. Jour. Gen. Physiol., **15**, 351–361.

PRECIPITINS FOR THE TUBERCULIN PROTEINS OF ACID-FAST BACTERIA[1]

JANET McCARTER, ELIZABETH M. KANNE, AND E. G. HASTINGS

Department of Agricultural Bacteriology, University of Wisconsin

Received for publication October 10, 1938

INTRODUCTION

Seibert (1930) found that the tuberculin proteins of the three varieties of tubercle bacillus could be distinguished by the precipitin test. If this method could be adapted for identifying tubercle bacilli, it would be preferable to animal inoculation. The behavior of the tuberculin proteins[2] of acid-fast bacteria in the precipitin test is interesting not only as a possible means of identification of the bacilli, but also because the proteins and their derivatives are apparently the active agents in tuberculins.

METHODS

The cultures investigated included one strain of the human tubercle bacillus; one strain of the bovine; five strains of the avian, three isolated from chickens, one from a hog, and one from a cow; one strain of Johne's bacillus; and two cultures of so-called saprophytic acid-fast bacteria, one isolated from a cow and one from a hog.[3] After the growth on the synthetic medium of

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation and is published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] By "tuberculin proteins" we mean proteins in culture filtrates as distinguished from proteins isolated directly from cells.

[3] The human strain was obtained from the Bureau of Animal Industry and has been used in our laboratory for making Old Tuberculin for twenty years. The bovine strain, isolated by Traum, was obtained from the Cornell Veterinary College. The avian strains, the Johne's bacillus, and the two saprophytic strains were all isolated in this laboratory. The Johne's bacillus grows without phlei cells. See discussion for description of saprophytic strains.

Dorset and Henley (1934) had autolyzed, usually three months after inoculation, the cells were removed by a paper and then by a Mandler filter. To the filtrate was added an equal volume of one-per-cent phenol. It was then concentrated to about one-tenth of the original volume by ultrafiltration and washed with 0.5 per cent phenol until a $BaCl_2$ test for sulphate was negative. Concentration was continued until the solution contained from 0.5 to 1.0 mgm. of protein per cubic centimeter. It was then filtered through a Berkefeld filter and handled aseptically thereafter. The amount of protein in the solution was estimated according to Seibert's (1928) method: precipitation of a 5-cc. sample with 5 cc. of 20-per-cent trichloracetic acid, centrifugation until a constant volume of precipitate was obtained, and computation for protein content from Seibert's determination on a human tubercle bacillus protein that 1 cc. of precipitate contains 0.0674 gram of protein. Nitrogen determinations on some of the protein solutions, by a modification of the micro-Kjeldahl method involving Nesslerization and colorimeter readings, showed from 10.5 to 12.5 per cent more protein than was estimated by Seibert's standardization method. As a precaution against one protein contaminating another, the Mandler and Berkefeld filters were cleaned after each use by soaking over night in a hot 0.5-per-cent sodium hydroxide solution; all glassware was cleaned with chromic acid cleaning solution, and new ultrafilters were used for each protein.

The precipitins were incited in rabbits by the subcutaneous injection of the antigens in solution, 3 to 5 cc. being given at intervals of two to three days until a total of about 25 mgm. of protein had been injected. In the first part of the work the rabbits were bled three weeks after the last injection, but later it was found that the sera taken one week after the last injection showed more antibody.

In making the precipitin tests, 0.1 cc. of the antigen solution was layered with a capillary pipette on 0.1 cc. of the antiserum. The antigen was tested in strengths starting from one gram of protein in 1250 or 2500 cc., each successive dilution being twice that of the preceding. Dilutions were usually made with 0.2

per cent salt solution, although concentrations of from 0.2 to 0.9 per cent gave no apparent differences in the results. Readings for rings were made after two hours at 37°C. and for precipitate after an additional 12 or more hours in the icebox. A lighting device described by Kanne and McCarter (1939) was used in examining for rings and precipitate.

In carrying out the precipitin absorption tests, preliminary tests were made with a constant volume of serum and varying quantities of antigen to determine the ratio necessary for the maximum amount of precipitate. In making the absorptions, apparently as much precipitate was formed if incubation was carried out wholly at 0° as if carried out partially at 37°.

RESULTS

The results of the precipitin tests with undiluted antisera and the tuberculin proteins of the various acid-fast bacteria are given in table 1. The tests with the human and bovine antisera are not conclusive, since the proteins of only one strain of each of the three varieties of tubercle bacilli were tried. However, these results agree with Seibert's (1930) in that the proteins of the three varieties of tubercle bacilli and of the two saprophytes were all distinct. The avian protein, even though it reacted in nearly as high a dilution as the human protein with the human antiserum, could be distinguished easily because it gave much less precipitate in corresponding dilutions. With the avian and Johne's antisera, the avian tubercle bacilli from the different sources and the Johne's bacillus could not be distinguished from each other, but could be distinguished from the human and the bovine types. Although the bovine protein reacted in high dilutions with the avian and Johne's antisera, in corresponding dilutions much less precipitate was obtained with the bovine protein than with any of the avian proteins or the Johne's protein. Apparently comparable amounts of precipitate with Johne's and avian proteins were obtained in both Johne's and avian antisera.

Tables 2 and 3 show attempts to differentiate the Johne's and avian proteins by precipitin absorptions and by antiserum

TABLE 1

Precipitin tests with tuberculin proteins and their antisera

ANTISERUM (UNDILUTED)	ANTIGEN TITER*									
	Human t.b.	Bovine t.b.	Avian t.b. 1 (chicken)	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus	Saprophytic acid-fast (cow)	Saprophytic acid-fast (hog)
Human	10,000		5,000						0	0
Bovine	10,000	40,000	5,000						2,500	0
Avian 1	0		20,000						0	0
Avian 2	10,000	80,000	160,000	320,000	80,000	40,000	160,000	320,000	20,000	10,000
Avian 4	0	40,000	80,000	80,000	40,000	80,000	80,000	80,000	10,000	
Johne's	10,000	80,000		160,000	160,000	80,000	160,000	160,000		5,000
Saprophyte (cow)	0	0	5,000						40,000	0

* The titer is given as the highest dilution (expressed as cubic centimeters of solution containing 1 gram of protein) of the protein solution showing a ring or precipitate as compared with a control of antiserum and salt solution. The titers for rings and precipitate were usually the same, although, when the lighting device was used, definite rings could sometimes be seen with higher dilutions than showed precipitates.

TABLE 2

Precipitin absorptions

TUBERCULIN PROTEIN WITH WHICH ANTISERUM ABSORBED	ANTIGEN TITER					
	Human t.b.	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Johne's bacillus
Antiserum for the tuberculin protein of avian t.b. 4 (diluted 1 to 4)						
Salt solution control	0	640,000	320,000	320,000	640,000	640,000
Avian t.b. 4		0	0		0	0
Avian t.b. 3		0	0	0	0	0
Bovine t.b		0	10,000	80,000	160,000	80,000
Antiserum for the tuberculin protein of the Johne's bacillus (diluted 1 to 4)						
Salt solution control	5,000	160,000	80,000	160,000	80,000	320,000
Avian t.b. 4	0	0	0	0	0	0
Avian t.b. 3	0	0	0	0	0	0
Bovine t.b		0	20,000	80,000	80,000	160,000

TABLE 3

Precipitin tests with diluted antisera

ANTISERUM (DILUTED 1 TO 32)	ANTIGEN TITER					
	Bovine t.b.	Avian t.b. 2 (chicken)	Avian t.b. 3 (chicken)	Avian t.b. 4 (cow)	Avian t.b. 5 (hog)	Johne's bacillus
Avian 4	0	40,000	80,000	320,000	0	80,000
Johne's	0	0	40,000		160,000	160,000

dilution. In the precipitin absorption tests, absorption of both avian and Johne's antiserum with bovine protein removed only bovine-reacting precipitins, while absorption with avian protein removed bovine, avian, and Johne's precipitins. The Johne's protein never completely absorbed precipitins for either the avian or Johne's proteins and is, therefore, not included in the table. When the antisera were diluted 1 to 32, as in table 3, the bovine-protein-reacting precipitins were eliminated but the Johne's and avian were still not distinguishable. Further dilution eliminated both Johne's and avian precipitins.

DISCUSSION

That the proteins are the active antigens in tuberculins has been assumed in the interpretation of our results. This assumption is made on the basis of the finding by Seibert and Munday (1931) that the antigenicity of tuberculins in the precipitin test is correlated with high nitrogen content and large molecular size rather than with carbohydrate content, although polysaccharide is combined in some way with the protein in ultrafiltered tuberculins. This polysaccharide is removed from the protein derivatives in the P.P.D. (Purified Protein Derivative) tuberculin by repeated precipitation of the protein solution with trichloracetic acid. Since we wished to alter the protein as little as possible, and were interested in finding a test for identifying acid-fasts which would involve as few manipulations as possible, we did not remove the carbohydrate from the protein solution.

On the basis of our results, human, bovine, and avian tuberculin proteins could be distinguished from each other by the precipitin test. The separation of human from avian protein is evident from table 1, since the avian always gave much higher titers with avian and Johne's antisera. The bovine was separated from the avian on the basis of a much higher titer with bovine antiserum and much less precipitate with avian or Johne's antiserum in comparable dilutions. Also, the bovine protein did not absorb all the avian or Johne's precipitins from either the avian or the Johne's antiserum but only reduced them slightly. (We did not test the capacity of the bovine protein to absorb com-

pletely the bovine antiserum, and therefore would not draw conclusions from the absorption test alone, since the antigen in question has not been shown to absorb completely its own or a heterologous antiserum.)

The proteins of the three avian strains from chickens, the strain from the cow, and the strain from the hog, all behaved so nearly alike in the precipitin test as to be indistinguishable. Some variations do occur in the behavior of the different avian proteins in the antisera for the avian tubercle bacillus strains 2 and 4, and in the diluted antiserum for strain 4. These variations can be attributed to varying amounts of different proteins in the same tuberculin or of different reactive groups on any one protein. Seibert, Pedersen, and Tiselius (1938) have found that even the P.P.D. tuberculin contains several proteins or protein derivatives of different molecular weights. The bovine protein apparently reacts in avian antisera with the antibody for only part of the proteins or reactive groups.

The surprising result was that the Johne's bacillus protein could not be distinguished from the avian tubercle bacillus proteins in either undiluted or diluted antisera. The precipitin absorption tests gave corroborative evidence that the tuberculin proteins of the Johne's bacillus and of avian tubercle bacilli are closely similar, since the proteins of the avian strains 3 and 4 removed all precipitins from the antisera for the Johne's protein and for the avian (strain 4) protein.

The Johne's bacillus and the avian tubercle bacillus have widely different cultural characteristics and pathogenic capacities. Evidently, however, they have a common protein and therefore cannot be identified by the precipitin test. Consequently the test can not be used by itself to identify an unknown acid-fast, but can be used to give evidence supplementary to the cultural and pathogenic characteristics. Thus, the culture of avian tubercle bacilli from the cow, designated as strain 4, reacts as an avian or Johne's strain in the precipitin tests; has the cultural characteristics of the avian tubercle bacillus; is pathogenic for rabbits and not for guinea pigs; but does not produce pro-

gressive tuberculosis in chickens (a full description of this culture will be published).

The similarity of the tuberculin proteins of the Johne's bacillus and the avian tubercle bacillus is interesting because it gives a stronger basis for the use of avian tuberculin in the diagnosis of Johne's disease, which Hagan and Zeissig (1927–28) have advocated on the basis of clinical findings. It had been thought that this might be because both avian tubercle bacilli and Johne's bacilli are so often isolated from animals having Johne's disease. On the basis of the precipitin test, the common proteins probably account for the success of the diagnostic test.

That neither of the two saprophytes tested is a variety of tubercle bacillus was established by the precipitin test. The one microorganism was isolated from a no-visible-lesion tuberculin-reacting cow. Injection of this culture sensitized another cow to human tuberculin, and Feldman (1933) found that it sensitizes chickens to avian tuberculin. The other saprophyte was isolated from a hog, from lymph nodes showing caseous pinhead-sized tubercles. Neither culture is pathogenic for rabbits, chickens, or guinea pigs, and both cultures have quite similar cultural characteristics. The protein of the saprophyte from the hog does not give any cross reaction in the antiserum for the protein of the saprophyte from the cow; indicating that the two saprophytes are different species.

The occurrence of cross-reactions with the avian tuberculin protein in the antisera for the mammalian (human and bovine) tuberculin proteins, and with the mammalian proteins in the avian antisera, is in agreement with the findings that all human beings sensitive to mammalian tuberculin react to avian tuberculin (McCarter, Getz, and Stiehm (1938)), and that cattle infected with avian tubercle bacilli react to mammalian tuberculin (McCarter, Beach and Hastings (1937)).

Since the tuberculin proteins of the three varieties of tubercle bacilli, of the Johne's bacillus, and of the two saprophytes all reacted in more than one heterologous antiserum, it is doubtful whether a tuberculin test with any of the available tuberculins

can be used to diagnose for infection with a specific acid-fast microorganism; i.e., whether a reaction to a specific tuberculin necessarily means that the animal tested has been infected with the acid-fast from which the tuberculin was made.

CONCLUSIONS

1. The tuberculin proteins of the human, the bovine, and the avian tubercle bacillus are distinguishable by the precipitin test.

2. The tuberculin proteins of the avian tubercle bacilli isolated from the chicken, the cow, and the hog, and the protein of the Johne's bacillus can not be differentiated by the precipitin test.

3. The tuberculin proteins of two so-called saprophytic acid-fast bacteria, one isolated from the cow and one from the hog, are distinguishable from each other and from the proteins of the tubercle bacilli and of the Johne's bacillus.

4. The precipitin test with tuberculin proteins as antigens is useful in identifying unknown acid-fast bacteria when considered in conjunction with cultural and pathogenic characteristics. Further purification of the protein solutions seems necessary before the precipitin test can replace other methods of identification of acid-fasts.

REFERENCES

DORSET, M., AND HENLEY, R. R. 1934 A synthetic medium for *B. tuberculosis*, with a description of the method of producing tuberculin. U. S. D. A., Bur. of Anim. Indus., mimeographed material.

FELDMAN, W. H. 1933 The sensitivity of chickens to tuberculin following exposure to different varieties of acid-fast bacilli. Jour. Amer. Vet. Med. Assoc., **83**, 344–357.

HAGAN, W. A., AND ZEISSIG, A. 1927–28 Johnin versus avian tuberculin as a diagnostic agent for paratuberculosis (Johne's disease) of cattle. Ann. Rpt. Cornell State Veterinary College, 172–189.

KANNE, ELIZABETH M., AND McCARTER, JANET R. 1939 A device for viewing precipitin reactions. Jour. Lab. and Clin. Med., in press.

McCARTER, JANET, HASTINGS, E. G., AND BEACH, B. A. 1937 The presence of avian tubercle bacilli in the lymph-nodes of cattle. Jour. Amer. Vet. Med. Assoc., **90**, 619–626.

McCARTER, JANET, GETZ, H. R., AND STIEHM, R. H. 1938 A comparison of intracutaneous reactions in man to the purified protein derivatives of several species of acid-fast bacteria. Amer. Jour. Med. Sci., **195**, 479–493.

SEIBERT, FLORENCE B. 1928 An improved and simplified method for making a standard undenatured tuberculin of any desired strength and a method of chemical assay. Jour. Biol. Chem., **78**, 345-362.

SEIBERT, FLORENCE B. 1930 Precipitin tests and differentiation of various tuberculin and timothy-bacillus proteins. Amer. Rev. Tubere. **21**, 370-382.

SEIBERT, FLORENCE B., AND MUNDAY, BETTY 1931 Analysis of the colloidal components of tuberculin with special reference to the relation of protein and carbohydrate. Amer. Rev. Tuberc., **23**, 23-40.

SEIBERT, FLORENCE B., PEDERSEN, KAI O., AND TISELIUS, ARNE 1938 Molecular weight, electrochemical and biological properties of tuberculin protein and polysaccharide molecules. Trans. 34th Ann. Meet. Natl. Tuberc. Assoc., in press.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., FEBRUARY 21, 1939

OXIDATION OF PERSEITOL BY ACETOBACTER SUBOXYDANS. *E. B. Tilden*, Division of Chemistry, National Institute of Health, Washington, D. C.

Perseitol (α-d-mannoheptitol), obtained from the seeds of the avocado, was oxidized by Bertrand, in 1908, to a crystalline ketoheptose (perseulose) by using the alcohol as carbon source in yeast extract cultures of his "sorbose bacterium" (*Acetobacter xylinum*). Bertrand obtained 45% yields of the sugar. The preparation of perseulose in quantity for chemical studies was the object of the present work.

In the bacterial method of preparation of sorbose, Kluyver and de Leeuw's *Acetobacter suboxydans* had recently been found to give better yields than *A. xylinum*. It also grows in a homogeneous suspension in culture. While the bacteria oxidize a commercial sorbitol syrup nearly quantitatively in the presence of 0.5% yeast extract alone, only 30% perseitol was oxidized under the same conditions. However, complete oxidation was obtained by addition to the yeast extract of a small amount of glucose (0.05%), together with phosphate buffer. The optimum pH for the reaction was 6.0–6.8. The glucose-phosphate-yeast extract appears to be a suitable medium for the oxidation of other pure sugar alcohols by *A. suboxydans*, as judged from preliminary tests in which the presence of reducing sugar was determined by Shaffer Hartmann tests.

EFFECT ON FACTORY CANE JUICES AND SIRUPS OF LEUCONOSTOC MESENTEROIDES ISOLATED FROM FROST DAMAGED LOUISIANA SUGARCANE OF THE 1937 CROP. *M. A. McCalip*, Carbohydrate Research Division, and *H. H. Hall*, Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture.

The gum-producing microörganism, *Leuconostoc mesenteroides*, was isolated from the 1937 crop of Louisiana frozen sugarcane. The gum dextran, produced by the microörganism at the expense of sucrose, greatly increases the viscosity of juice and sirup from the cane, retards filtration and crystallization and introduces difficulty and errors in sucrose determinations. Growth of a pure culture in non-buffered 10 per cent sucrose solution at 20°C. results in a loss of sucrose, rapid increase in acidity and increase in reducing sugars in which glucose predominates. When the microörganism was cultured in a sugar solution buffered to pH 7.5, the viscosity was increased several fold and crude gum was formed to the extent of approximately 50 per cent of the original

sucrose content. d-Mannitol was recovered from the solutions which underwent viscous fermentation.

A Basidiomycete Culture from Sputum. *C. W. Emmons*, Division of Infectious Diseases, National Institute of Health, Washington, D. C.

Notes on Recent Books of Interest to Bacteriologists. *M. C. Leikind*, Library of Congress.